Optimization of β-Glucosidase activity in recombinant *Saccharomyces cerevisiae* strains

by

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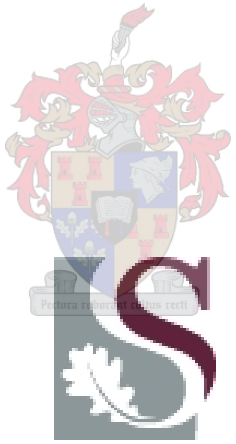

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at Stellenbosch University.

December 2007

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Ntanganedzeni Ranwedzi **Date**

SUMMARY

Wine is a complex medium. Wine aroma, flavour and colour are important quality factors, but these can be influenced by many factors, such as grape-derived compounds that exist as free volatiles and also as glycosidically bound. The chemical composition of wine is determined by factors such as grape variety, geographic position, viticulture condition, microbial ecology of the grape and the winemaking process. The varietals aroma is determined by both the volatile and the non-volatile compounds, such as monoterpenes, norisoprenoids and benzene derivatives, which are naturally present in the wine. Monoterpenes are very important in the flavour and aroma of grapes and wine. They can be found in grapes and wine either in the free, volatile and odorous form, or in the glycosidically-bound, non-volatile and non-odorous form. The ratio of glycosidically-bound compounds to free aroma compounds is very high in the Gewürztraminer, Muscat and Riesling cultivars in particular.

The glycosidic bonds can be hydrolysed either by the acid method or by using enzymes. The acid method is disadvantageous because it can modify the monoterpenes, whereas enzymatic hydrolysis has the advantage of not modifying the aroma character. The enzyme method of breaking the glycosidic bonds occurs in two successive steps: initial separation of glucose from the terminal sugar by a hydrolase (α-L-arabinofuranosidase, α-L-rhamnosidase or β-apiosidase, depending on the aglycone moiety), followed by the breaking of the bond between the aglycone and glucose by β-glucosidase.

The enzyme β-glucosidase can be obtained from many plant (*Vitis vinifera*), bacterial, yeast or fungal sources. Most of the enzymes produced by these sources are not functional under the winemaking conditions of low pH, low temperature, high glucose and high ethanol content. However, β-glucosidases from fungal origins, particularly from *Aspergillus* spp., are tolerant of winemaking conditions.

The idea of using the β-glucosidase gene from the fungus *Aspergillus kawachii* (*BGLA*), which is linked to the cell wall and the free β-glucosidase, was to determine if anchoring the enzyme to the cell wall will increase the activity of the enzyme compared to the free enzyme. Four plasmids, pCEL 16, pCEL 24, pDLG 97 and pDLG 98, were used in this study. *BGLA* that was cloned into the plasmids pCEL 24 and pDLG 97 was linked to CWP2, and in pDLG 98 it was linked to AGα1 anchor domains. All the plasmids were genome-integrated and expressed in the reference strain *Saccharomyces cerevisiae* 303-1A. All the transformants were grown in 2% cellobiose and showed higher biomass production compared to the reference strain. β-Glucosidase activity was also assayed and transformed strain W16 showed a fourfold increase in activity compared to the reference strain. There was no significant increase in the activity of the other transformed strains, W24, W97 and W98. Enzymatic characterisation for optimum pH and temperature was

done – for all strains the optimum pH was 4 and the optimum temperature was 40ºC.

The recombinant strains together with the reference strain were used to make wine from Gewürztraminer grapes. The levels of numerous monoterpenes were enhanced in the resultant wines. The concentration of nerol was increased fourfold, that of citronellol twofold, and geraniol was 20% higher than in the wild type. There was also an increase in the levels of linalool and α-terpinol, but this was not significant. In wines produced with W97, W98 and W24, monoterpene levels did not show a significant difference.

In future, the expression of the W16 expression cassette in an industrial wine yeast strain could be performed. In combination with the production of enzymes such as α-arabinofuranosidase, α-rhamnosidase and β-apiosidase, which are involved in the first step of enzymatic hydrolysis, this wine strain could release the bound monoterpenes and enhance the aroma of the wine.

OPSOMMING

Wyn is ‘n komplekse medium. Wynaroma, -geur en -kleur is belangrike kwaliteitsfaktore, hoewel hierdie kwaliteite deur verskeie faktore beïnvloed kan word, soos druifafgeleide verbindings wat as vry vlugtige stowwe teenwoordig kan wees of glikosidies gebind is. Die chemiese samestelling van wyn word bepaal deur faktore soos druifvariëteit, geografiese ligging, wingerdkundige toestande, mikrobiese ekologie van die druif en die wynbereidingsproses. Die variëteitsaroma word bepaal deur vlugtige en nie-vlugtige verbindings, soos monoterpene, norisoprenoïede en benseenderivate, wat natuurlik in die wyn voorkom. Monoterpene is baie belangrik vir die geur en aroma van druiwe en wyn. Monoterpene is teenwoordig in die druiwe en wyn in vry, vlugtige en geurige, of in glikosidiesgebinde, nie-vlugtige en nie-geurige vorms. Die verhouding van glikosidiesgebonde verbindings tot vry aromaverbindings is baie hoog, veral in die Gewürztraminer-, Muscat- en Riesling-kultivars.

Glikosidiese verbindings kan deur óf die suurmetode óf die ensiemmetode gehidroliseer word. Die nadeel van die suurmetode is dat dit monoterpene kan modifiseer, terwyl die ensiemmetode die voordeel het dat dit nie die aromakarakter modifiseer nie. Die ensiemmetode waarmee die glikosidiese verbinding afgebreek word, vind in twee opeenvolgende stappe plaas: aanvanklike skeiding van glukose van die terminale suiker deur ‘n hidrolase (α-L-arabinofuranosidase, α-L-ramnosidase of β-apiosidase, afhangende van die aglikoongedeelte), gevolg deur die verbreking van die verbinding tussen die aglikoon en glukose deur β-glukosidase.

Die β-glukosidase-ensiem kan vanaf ‘n verskeidenheid plant- (*Vitis vinifera*), bakterie-, gis- en swambronne verkry word. Die meerderheid van die ensieme wat deur hierdie bronne geproduseer word, is nie onder die wynbereidingstoestande van lae pH, hoë temperatuur, hoë glukose en hoë etanol funksioneel nie. β-Glukosidase vanaf ‘n swamoorsprong, veral vanaf *Aspergillus*-spesies, kan egter wynbereidingstoestande verdra.

Die idee agter die gebruik van die β-glukosidasegeen afkomstig van die swam *Aspergillus kawachii* (*BGLA),* wat aan die selwand en die vry β-glukosidase gekoppel is, was om te bepaal of die aktiwiteit van die ensiem in vergelyking met dié van die vry ensiem verhoog sou word indien die ensiem aan die selwand geanker is. Vier plasmiede, pCEL 16, pCEL 24, pDLG 97 en pDLG 98, is in hierdie studie gebruik. *BGLA*, wat in die plasmiede pCEL 24 en pDLG 97 gekloneer is, is gekoppel aan CWP2, en in pDLG 98 is dit aan AGα1-ankergebiede gekoppel. Al die plasmiede is in verwysingsras *Saccharomyces cerevisiae* 303-1A genoomgeïntegreer en uitgedruk. Al die transformante is in 2% sellobiose gegroei en het hoër biomassaproduksie as die verwysingsras getoon. β-Glukosidase-aktiwiteit is ook geëssaieer en die getransformeerde ras W16 het ‘n viervoudige

verhoging in aktiwiteit in vergelyking met die verwysingsras getoon. Daar was geen noemenswaardige verhoging in die aktiwiteit van die ander getransformeerde rasse, W24, W97 en W98, nie. Ensimatiese karakterisering vir optimum-pH en -temperatuur is gedoen – vir al die rasse was die optimum-pH 4 en die optimumtemperatuur 40ºC.

Die rekombinante rasse, tesame met die verwysingsras, is gebruik om wyn met Gewürtztraminer-druiwe te maak. Die vlakke van talryke monoterpene is in die gevolglike wyne verhoog. Die konsentrasie van nerol is viervoudig verhoog, dié van sitronellol tweevoudig, en geraniol was 20% hoër as in die wilde tipe. Daar was ook ‘n verhoging in die vlakke van linaloöl en α-terpinol, maar hierdie verhoging was nie noemenswaardig nie. In wyne wat met W97, W98 en W24 gemaak is, het die monoterpeenvlakke nie ‘n noemenswaardige verskil getoon nie.

In die toekoms sal die uitdrukking van die W16-uitdrukkingskasset in ‘n industriële wyngisras uitgevoer kan word. In kombinasie met die produksie van ensieme soos α-arabinofuranosidase, α-ramnosidase, β-apiosidase, wat in die eerste stap van ensimatiese hidrolise betrokke is, sal hierdie wyngisras die gebonde monoterpene kan vrylaat en die aroma van die wyn kan verbeter.

Kha vhabebi vhanga ndi ri ndi khou livhuwa zwothe zwe vha ita,lufuno lwavho ndo lu vhona u tikedziwa hothe na u tutuwedziwa nahone ndi ha u livhuwa. Zwe vha ita ndi do dzula ndi tshi zwi humbula maduvha othe a vhutshilo hanga nahone ndi a kholwa nga linwe la maduvha ndi do kona u humisela murahu zwe vha ita.

Kha muzwala wanga Khavhatondwi ndiri naho ni songo vhona mafhelo a ngudo iyi, ndi khou livhuwa u tutuwedziwa,muya wanu u lale nga mulalo.

Serenity prayer

God grant me the serenity to accept the things I cannot change, the courage to change things I can, and the wisdom to know the the difference.

BIOGRAPHICAL SKETCH

Ntanganedzeni Ranwedzi was born in Lwamondo, Matatani (Venda) in Limpopo Province on 21 January 1983. She attended Maphuphe Junior Primary School, Matshele Higher Primary School and matriculated at Luvhai-vhai Senior Secondary School in 2000. Ntanganedzeni enrolled at the University of Venda (Univen) in 2001, and obtained a BSc in Microbiology and Biochemistry. In 2004 she obtained a BSc Hons degree in Biochemistry at the University of Limpopo. In 2005 she enrolled for MSc in Wine Biotechnology, Stellenbosch University.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

GOD ALMIGHTY, for giving me strength and good health and making it possible for me to complete this degree. (Phillipians 4:13; Luke 18:27; II Corithians 12:9)

MY FAMILY, my loving and caring parents (Maemu and Ntsengeni Ranwedzi) for their unconditional love, believing in me, and their support, I will always appreciate and be grateful. My siblings: sisters (Avhatakali, Nthuseni and Pfano) and brothers (Mmbulungeni and Livhuwani) for always supporting me through out my study. I love you guys.

Prof PIERRE VAN RENSBURG, for reading of the dissertation and for acting as my supervisor and all the invaluable discussions

Prof RICARDO R CORDERO OTERO, for invaluable discussions and critical reading of dissertation, being my supervisor and all the invaluable discussions

ANNEL SMIT and **VASUDEVAN THANVANTHRI GURURAJAN**, for technical assistance, invaluable discussions, and moral support; for that I will always be grateful and thank you guys, you are amazing.

KAROLIEN ROUX and **ANDREAS TREDOUX,** for the assistance with monoterpene analysis.

MY FELLOW RESEARCHERS at the **IWBT** and **IWBT STAFF**

THE NATIONAL RESEARCH FOUNDATION, **UNIVERSITY OF STELLENBOSCH** and **INSTITUTE OF WINE BIOTECHNOLOGY** for financial support

TO MY FRIENDS, thank you for all the support and the encouragement you have given me through out the years.

PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal *Food Microbiology* to which Chapter 3 will be submitted for publication.

Chapter 1	**General Introduction and Project Aims**
Chapter 2	**Literature Review** The enzyme β-glucosidase in wine biotechnology: An overview
Chapter 3	**Research Results** Improving β-glucosidase activity in recombinant *Saccharomyces cerevisiae* strains
Chapter 4	**General Discussion and Conclusions**

CONTENTS

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

β-Glucosidase, which is also known as β-D-glucoside glucohydrolase (E.C 3.2.1.21), is a group of enzymes that hydrolyses a variety of glycosides, including aryl- and alkyl-β-glucoside and ρ-nitrophenyl-β-D-glucoside, and disaccharides such as cellobiose. This enzyme also has a synthetic mechanism according to which it may be used in the synthesis of compounds such as oligosaccharides and glyco-conjugates. β-Glucosidase belongs to families 1 and 3, and is ubiquitous as it occurs in the entire living kingdom.

1.1.1 SOURCES AND APPLICATION OF β-GLUCOSIDASE

β-Glucosidase is found in all living kingdoms. This enzyme plays various roles in biology, including the degradation of cellulose biomass by fungi and bacteria and the degradation of glycolipids in mammalian lysozomes (Roubelakis-Angelakis 2001). In humans, β-glucosidase (glucocerebrosidase) is found that has potential in the development of therapeutic and diagnostic procedures that are useful in the treatment of Gaucher disease. In microorganisms, β-glucosidase hydrolyses cellobiose and short-chain oligosaccharides into glucose. These enzymes are of considerable industrial interest, not only as cellulose-degrading systems, but also in the food industry. β-Glucosidase from microorganisms also plays an important role in the enhancement of fruit and wine aroma through the liberation of monoterpene alcohols (Esen 1993). In plants, β-glucosidase has been implicated in a variety of key metabolic events and growth-related responses, ranging from defence against pathogens and herbivores, through the release of coumarins, thiocyanotes, terpenes and cyanide, to the hydrolysis of conjugated phytohormones (Esen 1993), as well as the cleavage of glycosylated flavonoids (Roubelakis-Angelakis 2001).

1.1.2 THE ROLE OF β-GLUCOSIDASE IN MONOTERPENE LIBERATION IN WINE

Monoterpenoids are 10-carbon compounds with strong sensory qualities. They are found widely in nature as chief constituents of many essential oils, making them important compounds in the flavour and fragments industry. A variety of food products, such as grapes, fruit juices and wines, also contain these compounds (Maicas and Mateo 2005). Among the monoterpenols, linalool, nerol, geraniol, α-terpinol and citronellol are more active from the olfactory point of view due to their low sensory threshold (Williams et al. 1982; Günata et al. 1985).

There are three types of monoterpenes: the free aroma compounds, which are volatile and odorous (Williams et al. 1980), the odourless polyols, which make no direct contribution to the aroma, and the non-odorous, non-volatile, glycosidically-conjugated form of monoterpenes. The ratios of bound to free monoterpenes range between 1 and 5 in the juice of mature grape cultivars of Muscat and Riesling, and up to 15 in the Gewürztraminer variety (Günata et al. 1988). Chardonnay and Sauvignon blanc have low concentrations of monoterpenes (Roubelakis-Angelakis 2001). Two methods can be

used to break down the glycosidic bond to release terpene –either by using acid or by using enzymes. The acid method has the disadvantage of causing the loss of the natural aroma profile of the product and changing the molecular arrangement of the monoterpenols (Rapp and Mandery 1986). The enzyme method is used the most because it does not modify the aroma character. Enzyme catalysis of monoterpenes occurs through two successive steps: first, glucose is separated from the terminal sugar by a hydrolase (α-L-arabinofuranosidase, α-L-rhamnosidase or β-apiosidase, depending on the aglycone moiety), and second, the bond between the aglycone and glucose is broken by β-glucosidase (Günata et al. 1988).

The efficiency of the hydrolysis of β-glucosidase depends, among other things, on the origin of the enzyme and the structure of the aglycone. Because plant β-glucosidases are inhibited by glucose, showing poor stability at the low pH, and high ethanol levels in wine, other sources have been used in order to enhance wine aroma (Günata et al. 1985; Aryan et al. 1987). Yeast shows more promise as a source of enzymes, as Delcroix et al. (1994) and Hernández et al. (2003) have shown that *S. cerevisiae* possesses β-glucosidase, but that the activity towards glycoside precursors is very limited. Bacterial β-glucosidase cannot be used because it is inhibited by 3% glucose, has a high pH optimum and an optimum temperature of about 65˚C, whereas a low pH and temperature are desired during winemaking. Because bacteria, yeast and plant β-glucosidases are not ideal for winemaking conditions, the focus changed to the use of fungal β-glucosidase. *Aspergillus* spp. produce stable enzymes, such as β-glucosidase, amylase, protease and hemicellulase, and it can grow in an acidic environment (Ohta et al. 1991; Ito et al. 1992; Mikami and Iwano 1988; Sudo et al. 1993). Fungal β-glucosidase from *Aspergillus* spp is also used because it is glucose tolerant, stable at low pH and highly active on cellobiose (Sternberg 1976).

PROJECT AIMS

The aim of this study was to optimise the efficiency of β-glucosidase encoded by the *BGLA* gene from *Aspergillus kawachii* expressed in *Saccharomyces cerevisiae*. The approaches used were as follows:

i) Testing the expression of β-glucosidase levels under different promoter regulations such as enolase (*ENO1*) and phosphoglycerate kinase (*PGK1*).

ii) Localising the enzyme to the cell wall using two cell wall anchor domains, CWP2 and AGα1.

iii) Microvinification using the recombinant yeasts to determine monoterpenes release.

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CHAPTER 2

LITERATURE REVIEW

The enzyme β-glucosidase in wine biotechnology: An overview

2.1 INTRODUCTION

Enzymes play an important role in the winemaking process. They play a role in both pre- and post-fermentation practices. There are at least 10 different enzymes that are involved in the fermentation kinetics that convert grape juice to wine. These enzymes are involved in improving wine quality through i) the clarification and processing of wine (pectinases, glucanases, xylanases, proteases); ii) the release of varietal aromas from precursor compounds (glycosidase); iii) the reduction of ethyl carbamate formation (urease); and iv) the reduction of alcohol levels (glucose oxidase) (Van Rensburg and Pretorius 2000). Commercial enzymes are expensive and many winemakers regard them as unnatural, the use of yeast that produce the enzyme(s) needed in wine making is essential. The use of exogenous enzymes from fungi, yeast and bacteria is also of importance. The focus of this literature review is the enzyme β-glucosidase, which contributes to the release of varietals aromas from glycoside compounds.

β-D-glucoside glucohydrolase (EC 3.2.1.21), commonly known as β-glucosidase, is a group of enzymes that hydrolyses a broad variety of glycosides, including alkyl- and aryl-β-D-glucoside and ρ-nitrophenyl-β-D-glucoside, and disaccharides such as cellobiose (Woodward and Wiseman 1982). There are many sources of β-glucosidase, namely plant glycosides, fungi, bacteria, yeast, mammals, etc. Their physiological functioning differs depending on the source and substrate specificity. In the case of yeast cells, the location (intracellular, extracellular or cell wall bound) of the β-glucosidases play a role in the functioning of this enzyme.

One of the compounds that contribute to wine aroma is monoterpene. Monoterpenes occur as free, volatile, odorous and non-glycosidically bound or as non-volatile, odourless and glycosidically bound or as polyols. The ratio of glycosidically bound is higher than the rest. It is therefore of importance to find a way to unleash the pool of grape-derived volatile aglycon and providing an enhancement of the flavour compounds in wine.

2.2 IMPORTANCE OF β-GLUCOSIDASE IN WINEMAKING

Winemaking is a microbiological process that involves different yeasts and lactic acid bacteria. *S. cerevisiae* is used as a starter culture to ensure a controlled fermentation and to yield wine of uniform quality with sensory attributes typical of each style of wine. Wine aroma is the collective outcome of an interaction between grape-derived compounds, those produced during fermentation and those produced during ageing (Hernández et al*.* 2003).

A good wine is determined by its colour, aroma and flavour. Wine aroma and flavour are influenced by grape-derived compounds that exist as free volatiles and as sugar-bound glycosides (Abbott et al*.* 1993; Williams et al*.* 1995). The chemical composition of wine is determined by factors such as grape variety, geographical position, viticultural

condition, microbial ecology of the grape and the winemaking practices (Cole and Noble 1997). Microorganisms play an important role in determining the chemical composition of wine. They affect the grape prior to harvest and during fermentation (Nykänen 1986; Lambrechts and Pretorius 2000).

Monoterpenoids are 10-carbon compounds with strong sensory qualities that are found widely in nature as chief constituents of many essential oils, making them valuable compounds in the flavour and fragrance industries. They are produced from geranyl pyrophosphate (GPP) precursors by higher plants (Figure 2.1) (Maicas and Mateo 2005), algae, fungi such as *Penicillium* (Larsen and Frisvad 1994, 1995), and even some yeast such as *Kluyveromyces lactis*, *Torulaspora delbrueckii* and *Ambrosiozyma monospora* (Klingenberg and Sprecher 1985).

There are volatile, free and odorous, and bound monoterpenes. Monoterpenes are mostly found in the bound form and can be released during the vinification process by the glycosidase enzyme produced by the grapes themselves or by microorganisms taking part in the process (Delcroix et al*.* 1994; Park and Noble 1993; Zoecklein et al*.* 1997). Hemingway et al. (1999) demonstrated the hydrolysis of one of the grape glycosides, neryl-β-D-glucoside, and the release of its flavour-active molecule, nerol. Monoterpenes are particularly abundant in aromatic grape varieties such as Muscat, Riesling and Gewürztraminer (Günata et al*.* 1990b). Different monoterpenes have distinct aromas, such as geraniol and nerol, which smell like roses, linalool, which smells like coriander, linalool oxide, which has a camphorous smell, and nerol oxide, which is vegetative (Simpson 1979).

The use of β-glucosidase in the wine industry is potentially interesting, because this enzyme can hydrolyse the monoterpene glycosides that occur naturally in wine, thus improving the aromatic structure of the wine (Williams et al*.* 1982a, Shoseyov et al*.* 1990). The addition of an exogenous aroma-liberating enzyme mix preparation is expensive and is viewed by scientists as a risky step. This has led to renewed interest in the production of uncontaminated β-glucosidase by *S. cerevisiae*, which will reduce the need to use a commercial cocktail of enzymes and will result in greater cost effectiveness. Pérez-González et al. (1993) reported that glycosylated flavour precursors were hydrolysed by *S. cerevisiae* when the β-1,4-glucanase from *Trichordem longibratum* was expressed in *S. cerevisiae*.

2.3. TYPES OF MONOTERPENES

Aroma, flavour and colour are important qualities factors in wine. The varietal aroma of wine is determined by volatile and non-volatile compounds, such as monoterpenes, norisoprenoids and benzene derivatives, which are naturally present in the wine. Terpenes are a class of compounds responsible for the varietal aroma of many fruits and their fermented products, such as wine and juice. Among the monoterpenols,

linalool, nerol, geraniol, α-terpinol and citronellol are more active from an olfactory point of view due to their low sensory thresholds (Williams et al. 1982b, Günata et al. 1985a).

There are three types of monoterpenes that exist in plant tissues, including the grape berry. The first is the free aroma compounds, which is volatile and odorous, and is commonly dominated by linalool, geraniol and nerol, as well as the pyran and furan forms of the linalool oxides. Depending on how the juice has been treated and on factors such as climate, many monoterpenes can be found in the following groups – citronellol, α-terpineol, hotrienol, nerol oxide, myrcenol ocimenol and other oxides, aldehydes and hydrocarbon. Ethyl ethers and acetate esters have also been found as free aroma compounds in wine (Williams et al. 1980). The second type is the free odourless polyols, which occur in the polyhydroxylated forms of the monoterpenes. These compounds (polyols) have significant features. Even though they make no direct contribution to the aroma, some of them are reactive and can break down and provide pleasant and potent volatiles, e.g. diendiol (3,7-dimethylocta-1,5-diene-3,7-diol) can produce hotrienol and nerol oxide (Williams et al. 1980). The final type are the non-odorous, non-volatile, glycosidically conjugated forms of monoterpenes (Williams et al. 1980), which are the most important because the ratio between bound and free monoterpenols ranges between 1 and 5 in the juice of mature grapes cultivars of Muscat and Riesling and up to 15 in the Gewürztraminer variety (Günata et al. 1988). They are even more abundant than the polyols (Mateo and Jimenez 2000). Non-aromatic cultivars such as Sauvignon blanc and Chardonnay have low concentrations of monoterpenes (Roubelakis-Angelakis 2001).

2.4 STRUCTURE OF GLYCOSIDES

Grape-derived aroma and flavour compounds are present as free volatiles and, in part, as sugar-bound precursors, including glycosides (Abbott et al. 1993). The compounds bound to a sugar molecule are known as aglycon and, in grapes, may be aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids or shikimic acid metabolites such as phenols (Abbott et al. 1993; Sefton et al. 1993, 1996; Winterhalter et al. 1990). Glycosides are found primarily in grape juice rather than in the skin or pulp fractions (Wilson et al. 1986). Bound glycosides exist mainly as monoglucosides or disaccharides (Salles et al. 1990; Voirin et al. 1990).

The aglycon moiety is always linked to β-D-glucopyranose. In the case of diglycosides, the glucose moiety is substituted with one of the following sugars: α-L-arabinofuranose, α-L-apiofuranose, α-L-rhamnopyranose, (Figure 2.2), β-D-glucopyranose, or α-L-xylopyranose. Glycosides are found primarily in grape juice rather than in the skin or pulp fractions (Wilson et al. 1986). Bound glycosides exist mainly as monoglucosides or disaccharides (Salles et al. 1990; Voirin et al. 1990).

Trivial names are given to some disaccharide substrates according to the plant species from which they are isolated: 6-O-α-L-arabinopyranosyl-β-D-glucopyranosides (vicianosides), 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides), 6-O-α-L-

apiofuranosyl-β-D-glucopyranosides, 6-O-α-L-glucopyranosyl-β-D-glucopyranosides (gentiobiosides), and 6-O-α-L-xylopyranosyl-β-D-glucopyranosides (primeverosides) (Guo et al. 1993; Stahl-Biskup et al. 1993; Williams et al. 1982a; Winterhalter and Skouroumounis 1997). The aglycone portion in the Riesling cultivar is frequently a terpenol (most notably linalool, nerol, geraniol or, in some cases, linalool oxides, terpene diols and triols (Günata et al. 1988; Williams et al. 1982b; Salles et al. 1990; Pérez-González 1993. Other aglycones include aliphatic or cyclic alcohols such as hexanol, 2-phenylethanol, benzyl alcohol, C13 norisoprenoids, phenol acids and some volatile phenols such as vanillan.

2.5 METHODS OF TERPENE GLYCOSIDE HYDROLYSIS IN WINE

Two methods could be used to break down the glycosidic bond to release terpenes: the acid method and the enzymatic method.

2.5.1 Acid method

Acid hydrolysis may split the alcohol aglycone and produce a reactive carbonation (Sefton 1998). The acidic way of releasing terpenes stimulates the reaction that takes place during the ageing of wine and different terpenic alcohols are produced in similar quantitative ratios. This method carries the disadvantage of losing the natural aroma profile of the product and changes the molecular arrangement of monoterpenols (Figure 2.3). Experiments done on both whole juice and monoterpene glycosides isolated from juice have shown significantly different patterns of volatile monoterpenes when each is hydrolysed at different pH levels. For example, (Williams et al. 1982b) have found that isomeric ocimenols appear to be formed hydrolytically in juice at pH 1, compared to linalool, nerol and geraniol being formed at pH 3. More acidic conditions cause an extensive rearrangement of monoterpenoids (Williams et al. 1982b). The acid hydrolysis method is closely dependant on the pH and temperature of the medium and the structure of the aglycone moiety. Glycosides of tertiary alcohols, such as linalool, linalool oxides and α-terpineol, are more readily hydrolysed than those of primary alcohols, such as geraniol and nerol, as has been observed in wine (Günata et al. 1988; Park and Noble 1993). Sefton (1998) found that the acidic hydrolysis of grape glycosides occurs when a protonated reagent breaks down the glycosyl bond between D-glucose and the aglycone, producing one molecule of water. The acid compounds found in wine can also cause such cleavage, but at normal wine pH (3.2-3.8), this reaction proceeds very slowly. One acid-hydrolysed reactant can yield a variety of volatiles that are potentially capable of affecting wine aroma, flavour and colour. Using the acidic method to breakdown the glycosidic bond has been shown to contribute varietal characteristics such as lime and honey to Chardonnay.

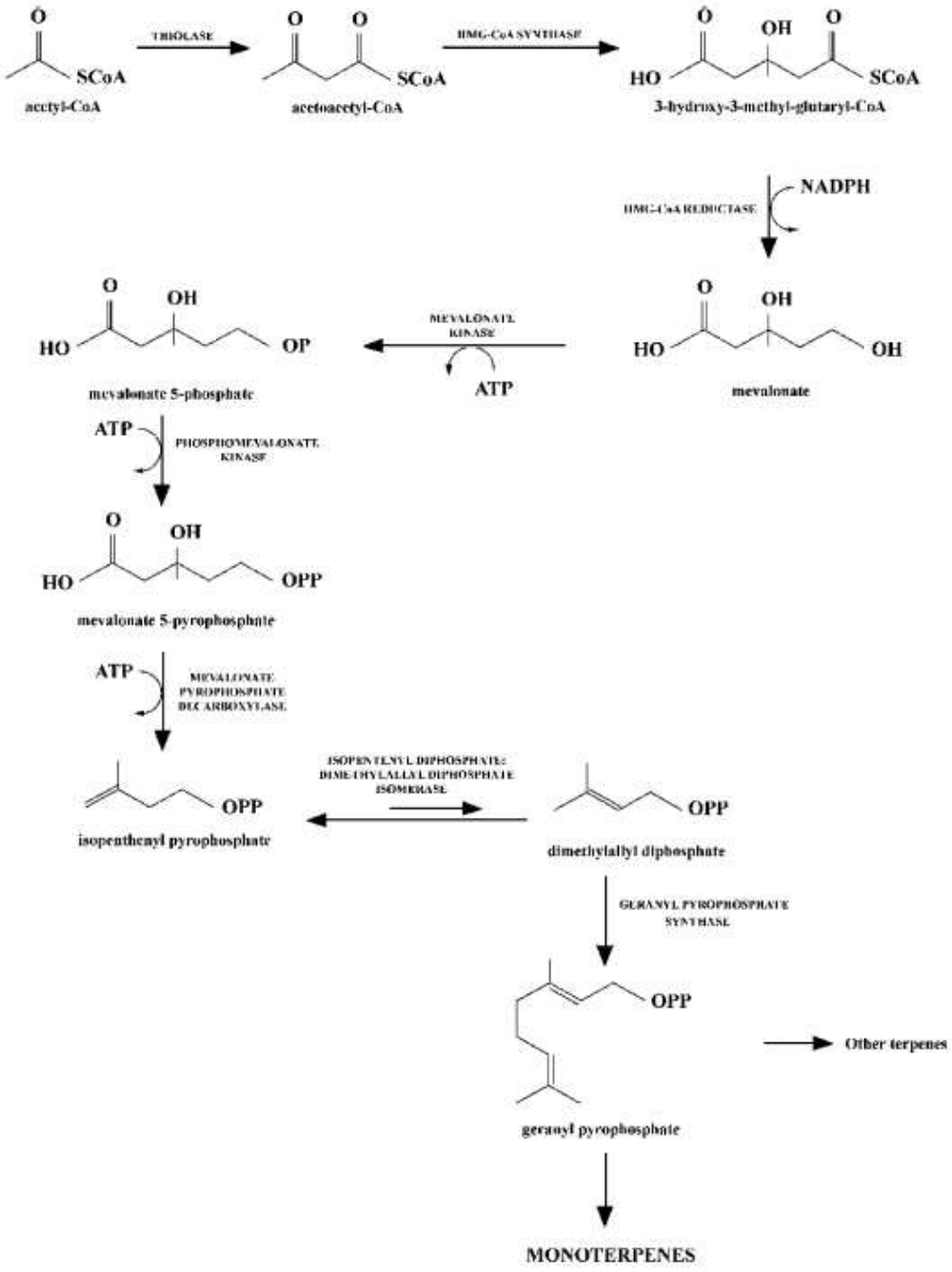

Figure 2.1 Mechanism of biosynthesis of monoterpenols in plants (Maicas and Mateo 2005)

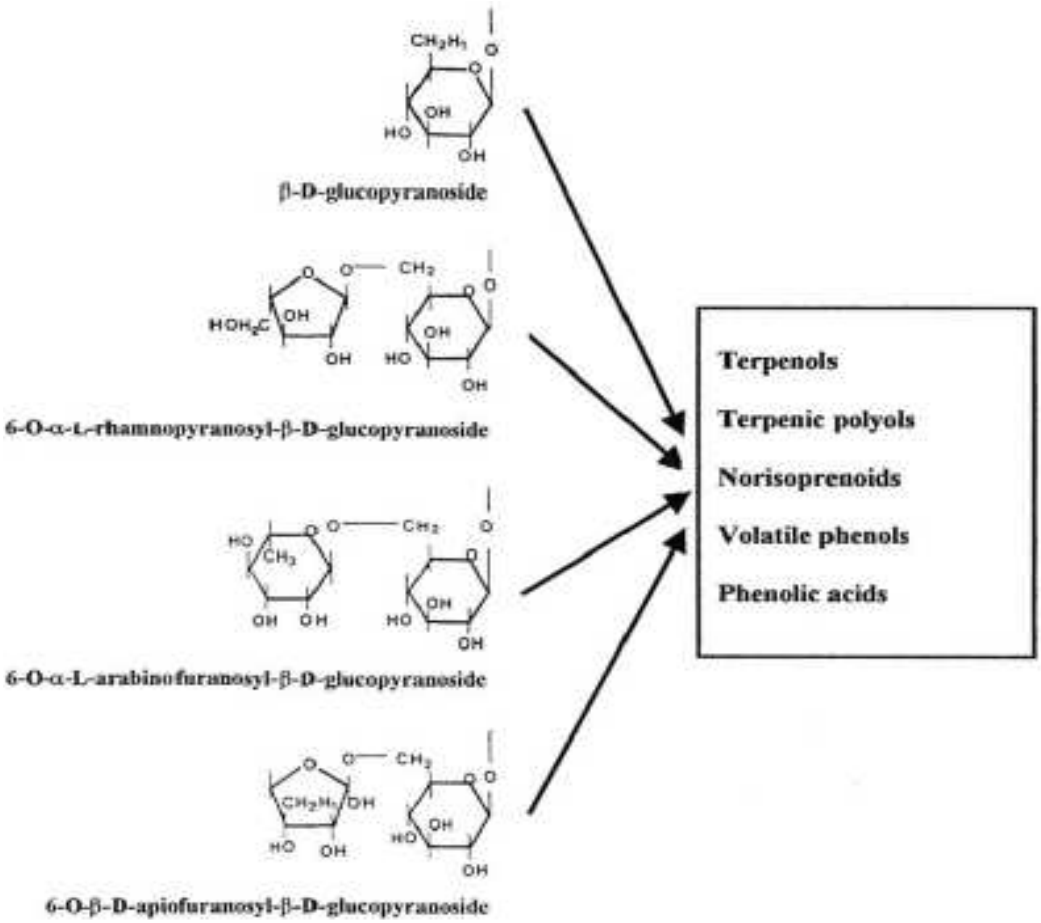

Figure 2.2 Structure of glycosidic aroma precursors from plants (Sarry and Günata 2004)

2.5.2 Enzyme method

The enzyme method is preferable to the acid method because it is more defined and does not change the aglycone (Winterhalter and Schreier 1995; Sefton 1998). Enzymatic catalysis of monoterpenyl glycosides occurs through two successive steps: first, glucose is separated from the terminal sugar by a hydrolase (α-L-arabinofuranosidase, α-L-rhamnosidase or β-apiosidase) and, second, the bond between the aglycone and glucose is broken by β-glucosidase (Figure 2.4).

The hydrolase enzyme needed to break the disaccharide bond can have specific or broad activity (Günata et al. 1988). In the case of monoglucosides, the β-glucosidase acts directly and, if the disaccharide moiety consists of a glucose unit, only the action of β-glucosidase is needed to complete the reaction. The efficient hydrolysis of monoterpenyl-β-D-glucoside by β-glucosidase depends on factors such as the origin of the enzyme and the structure of the aglycon. The β-glucosidase found in grapes promotes hydrolysis during fruit maturation, but has low activity and cannot liberate a large pool of aromatic precursors (Gueguen et al. 1997).

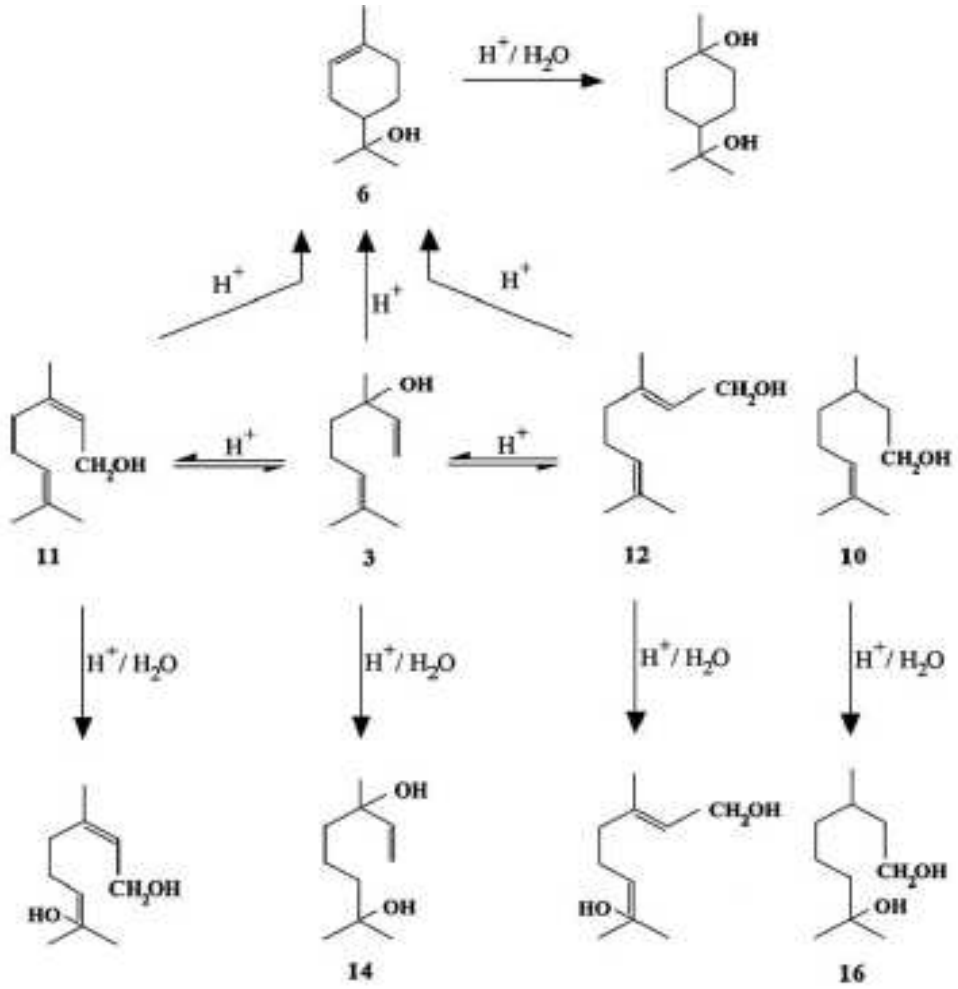

Figure 2.3 Figure 2.3 Acid catalysed rearrangement of monoterpenes. (Numbers below the structure represent the following monoterpenes: 3. Linalool, 6. α-terpinol, 10. Citronellol, 11. Nerol, 12. Geraniol, 14. Endiol, 16. Hydroxy-cityronellol) (Rapp and Mandery 1986).

2.6 SOURCES OF β-GLUCOSIDASE

There are many sources of β-glucosidase, such as plants, fungi, bacteria and yeast (Bhatia et al. 2002). Few β-glucosidases can be used under winemaking conditions, as they are not active at a low pH, high glucose concentration, high levels of ethanol, etc. The ideal β-glucosidase should be active at a low pH value between 2.5 and 3.8, at a high concentration of glucose (from 10 to 20%), and at an ethanol concentration of around 13% (Bothast and Saha 1997; Gueguen et al. 1997; Woodward and Wiseman 1982).

2.6.1 Plant glycosides

The plant β-glucosidases have been known for over 150 years, ever since the description of the action of emulsion (almond β-glucosidase). In plants, β-glucosidase activity has been implicated in a variety of key metabolic events and growth-related response, ranging from defence against some pathogens and herbivores, through the release of coumarins, thiocyanotes, terpenes and cyanide, to the hydrolysis of conjugated phytohormones (Esen 1993). *Vitis vinifera* (grapes) and *Humulus lupulus* (hops) are two plants that produce monoterpenes that have a significant value for the wine and brewing industries. There are two types of β-glucosidases in plants, namely β-D-glucosidase, which breaks down the O-linked β-glycosidic bonds, and β-δ-glucosidase (myrosinase), which catalyses the breakdown of S-linked β-glycosidic bonds (Bhatia et al. 2002). The occurrence of glycosidic flavour precursors in fruit was first reported in the grape berry, where it constitutes an important flavour potential (Günata et al. 1985a,b). Aryan et al. (1987); Günata et al. (1990a,b) found that grapes have β-glucosidase activity, but only low α-rhamnosidase, α-arabinosidase and β-xylosidase activities have been detected.

β-Glucosidase and exoglycosidase activities were found to increase during the ripening of grape berries (Aryan et al. 1987). β-Glucosidase of vegetal origin shows local activity on the monoglucosides of terpenes with a tertiary alcohol group (linalool, α-terpinol) and is only able to hydrolyse the monoglucosides of terpenes with a primary alcohol group, such as geranol, nerol and citronellol (Aryan et al. 1987; Günata et al. 1990b). Plant-produced β-glucosidases are characterised by a restricted specificity with respect to aglycon, they are not active between pH 3 to 4, and are inhibited by a glucose concentration over 1% (Aryan et al. 1987). These characteristics mean that grape β-glucosidases are not suitable to hydrolyse terpene glycosides in grape must or wine.

2.6.2 Fungal glycosidase

Species like *Aspergillus niger, Aspergillus awamori* and *Aspergillus kawachii* secrete large amounts of citric acid into the surrounding environment and cause it to be acidic. *A. kawachii* produces acid-stable enzymes such as amylase, protease, hemicellulase and β-glucosidase, which allow it to grow in an acidic environment (Ohta et al. 1991; Ito et al. 1992; Mikami and Iwano 1988; Sudo et al. 1993). Fungal β-glucosidases from *Aspergillus* sp. are mostly used to produce β-glucosidase because they are glucose tolerant and stable at low pH values. β-Glucosidase from *Aspergillus sp.* is also highly active on cellobiose, it can occur intracellularly and extracellularly, and has an optimum pH between 4.0 and 5.0 (Sternberg 1976). Fungal β-glucosidase has been expressed in eukaryotic systems such as *Trichoderma reesei* (Barnett et al. 1991), *Aspergillus sp*. and *Pichia pastoris* (Dan et al. 2000). The expression of a recombinant β-glucosidase from *A. kawachii* in *S. cerevisiae* has shown localised minor activity in the periplasmic space, whereas most recombinant fungal β-glucosidase is localised extracellularly.

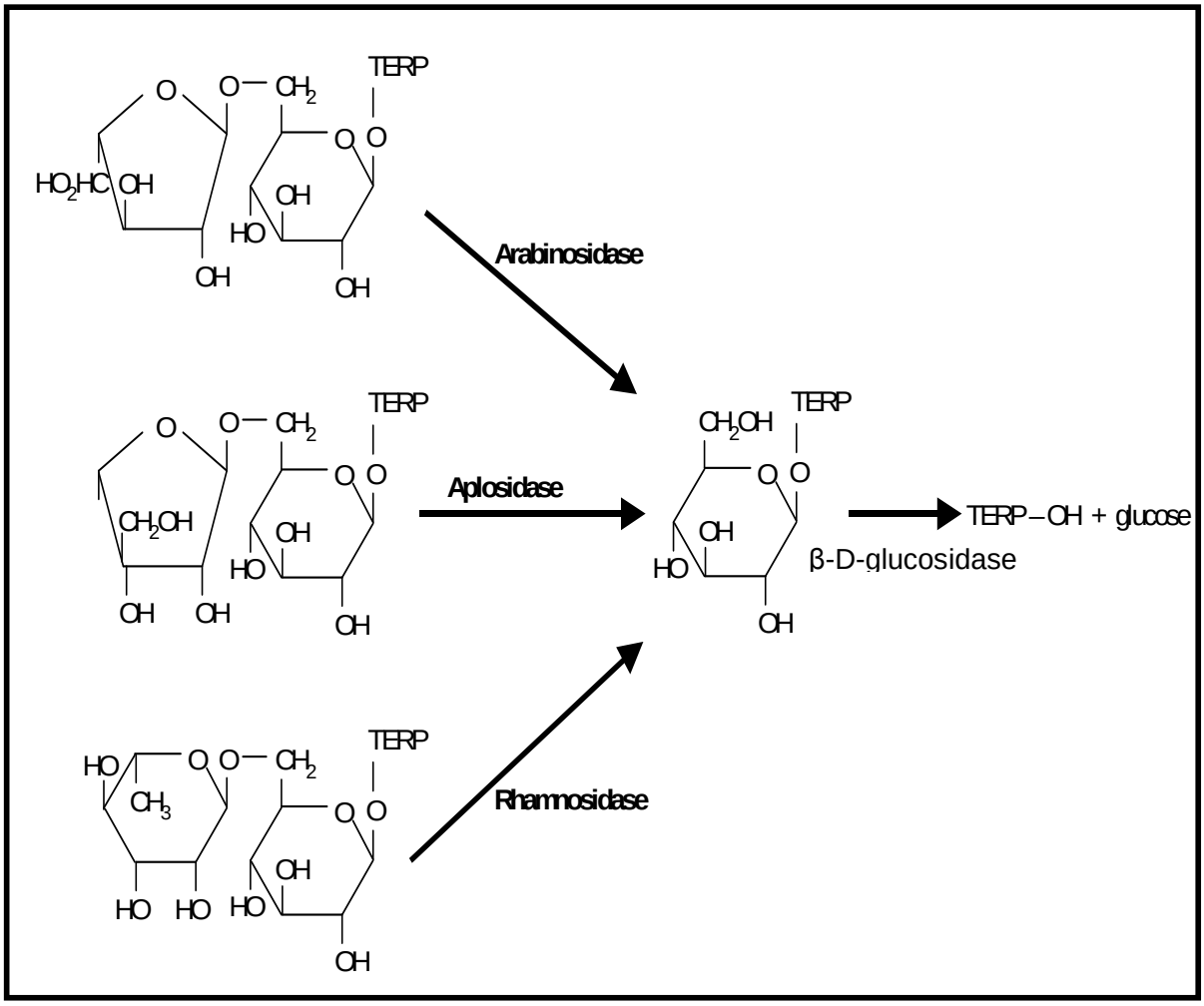

Figure 2.4 Sequential enzymatic hydrolysis of disaccharide flavour precursors (Günata et al. 1988)

2.6.3 Yeast glycosidase

Glycosidase activity is found primarily in the following grape must yeasts: *Hanseniaspora, Pichia, Candida, Saccharomycodes, Metschnikowia* and *Brettanomyces* sp., whereas it is rare in *S. cerevisiae* (Mateo and Di Stefano 1997). *S. cerevisiae* strains have low β-glucosidase activity and are inhibited under winemaking conditions (Winterhalter and Skouroumounis, 1997; Williams, 1993; Fernández et al. 2000). Günata et al. (1990b) found that the *Candida molischiana* and *Candida wickerhamii* yeasts produce β-glucosidase that has a low sensitivity to glucose and is active on range of non-specific aglycones. Rosi et al. (1994) found that a strain of *Debaryomyces hansenii* is capable of producing an exocellular β-glucosidase with activity inhibited by high ethanol and glucose concentrations, but unaffected by acid pH and low temperatures. *Brettanomyces* sp. has β-glucosidases that are highly active towards cellulose and hemicellulose, enabling them to hydrolyse large amounts of these polysaccharides, which are present in new barrels (Humphries et al. 1992).

2.6.4 Bacterial glycosidase

Lactobacillus plantarum has been found to hydrolyse oleuropein in brined Spanish olives via β-glucosidase production, producing simple compounds such as β-3,4-dihydroxyphenylethanol and an aglycone (Ciafardini et al. 1994), but this enzyme is inhibited by a 3% glucose concentration. *Clostridium thermocellum* produces β-glucosidase that has a great affinity for the aryl β-D-glycoside substrate with a pH optimum of 6 and at a temperature of 65℃ (Ait et al. 1979). However, this enzyme cannot be used under winemaking conditions because of both the low pH and temperature during winemaking. β-Glucosidase from *Bacillus polymyxa* has been expressed in *S. cerevisiae* (Adam et al. 1995) and was able to hydrolyse monoterpenic β-glucosidase. Boido et al. (2002) recently found that *Oenococcus oeni* involved in the malolactic fermentation of wine shows exoglycosidase and β-glucosidase activity, and the hydrolysis of glycosides has been reported during the enzymatic method. However this bacteria, does not increase the level of free aglycones. Sarry and Günata (2004) have found that the β-glucosidase gene from *Bacillus polymyxa* expressed in *S.cerevisiae* was able to hydrolyse monoterpenol β-glucosidase.

2.7 β-GLUCOSIDASE ENZYME: CLASSIFICATION AND MODE OF ACTION

β-Glucosidase glucohydrolases, commonly known as β-glucosidase, catalyse the hydrolysis of alkyl, aryl-β-glucosides, diglucosides and oligosaccharides.

2.7.1 Classification of β-glucosidase

The β-glucosidase enzymes occur in plants, fungi, yeast and bacteria. There is no defined method to classify these enzymes. Two classificatory methods appear in the literature, namely substrate specificity and nucleotide sequence identity (NSI) (Henrissat and Bairoch 1996). The substrate specificity method is divided into i) aryl-β-glucosidases, which act on aryl-glucosides, ii) true cellobiases, which hydrolyse cellobiose to release glucose, and iii) broad substrate specificity enzymes, which act on a wide spectrum of substrates. Many β-glucosidases are characterised as belonging to the broad substrate specificity group. On the other hand, the nucleotide sequence identity (NSI) method also came into use and one of the first classifications based on the available sequences proposed the grouping of this enzyme into two types, namely Type I and Type II β-glucosidase (Beguin 1990). In another scheme, proposed by (Rojas et al. 1995), β-glucosidases were divided into two subfamilies, subfamily A (BGA) and subfamily B (BGB). The earlier method has been replaced by the NSI scheme, which is accepted at present and is based on the sequence and folding similarities (hydrophobic cluster analysis, HCA) of these enzymes.

The classification scheme proposed for all glycosyl hydrolases, of which there are around 2000, has resulted in the recognition of 88 families. The nomenclature system is continuously being updated (Henrissat and Davis 1997). β-Glucosidase can also be

categorised as either family 1 or 3 of the glycosylhydrolases, with the exception of the glucosylceremidases (acid β-glucosidase), which belong to family 30. Family 1 have nearly 62 β-glucosidases from archaebacteria, plants and mammals, including 6-phosphoglycosidases and thioglucosidases. Many members of family 1 show a significant β-galactosidase activity (Bhatia et al. 2002). Family 1 β-glucosidases are also classified as members of the 4/7 super-family, with a common eight-fold β/α barrier motif (Kaper et al. 2000). The 4/7 super-family also includes enzyme like family 2 β-galactosidases, family 5 cellulases, family 10 xylanases, and family 17 barley glucanases (Jenkins et al. 1995). Family 3 glycosylhydrolases consist of 44 β-glucosidase and hexosaminidases of bacterial, mould and yeast origin. Enzymes of family 3 may further be subdivided into two classes, AB and AB'. At the molecular level, the genes of the family 3 glucosidase enzymes have five different regions: N-terminal residues, N-terminal catalytic domains, a no homologous region, a C-terminal domain of unknown function and C-terminal residues (Bhatia et al. 2002).

2.7.2 Mode of action

β-Glucosidases catalyse the hydrolysis of glycosidic linkages formed between the hemiacetal -OH group of cyclic aldose or glucose and the -OH group of another compound, such as sugar, amino-alcohol, aryl-alcohol or primary, secondary or tertiary alcohols. This reaction occurs in the following steps: firstly, during glycosylation an enzymatic nucleophile attacks the anomeric (C_1) centre of the substrate glycoside, resulting in the formation of a covalently linked α-glycosyl enzyme intermediate through an oxocarbonium ion-like transition state (Withers and Street 1989). The anomeric configuration at C_1 is then reversed, as shown in Figure 2.5, after which the second active residue of the enzyme serves as the acid base catalyst and donates H^+ to the glycosidic oxygen, thereby assisting in the departure of the aglycone group, or other glycones, as in disaccharides (Bhatia et al. 2002). The glycosyl-enzyme intermediate is hydrolysed via general base catalysed attack by water at the anomeric centre to release β-glucose as the product. The trans-addition of an -OH group results in the net retention of the β-anomeric configuration. The nucleophilic residue also acts as the leaving group in the deglycosylation step. The formation and hydrolysis of the enzyme's glycosyl intermediate occurs via an oxocarbonium ion-like transition state.

The reaction for the biosynthesis of glycoconjugates occurs either by reverse hydrolysis or by transglycosylation. The two-step mechanism employed by the retaining enzymes, such as β-glucosidase, allows these enzymes to transglycosylate. In reverse, the hydrolysis of the substrate has an H^+ in place of R (Figure 2.5). The enzyme glycosyl intermediate is intercepted by R`OH, where R is another sugar, yielding a disaccharide product. The reaction is under thermodynamic control (Bhatia et al. 2002). In the transglycosylation method, the substrate has R in the place of $H^+$ and is an activated anchor. The enzyme-glycosyl intermediate may be trapped as R`OH by a nucleophile other than water, such as aryl or alkyl alcohol, to produce a new glycoside. The efficiency of the formation of the product is determined by competition between

water and the acceptor R`OH for the enzyme-glycosyl intermediate. These reactions are under kinetic control (Bhatia et al. 2002).

2.8 FACTORS AFFECTING β-GLUCOSIDASE ACTIVITY

The conditions found in wine may severely inhibit the production and activity of enzymatic hydrolases. The β-glucosidase can be inhibited by: low pH, temperature, oxygen, high glucose concentration, ethanol, and phenols (Günata et al. 1994). Inhibition of β-glucosidase production and activity is related to the organism that expresses it (Aryan et al. 1987; Delcroix et al. 1994; Leclerc et al. 1984; Rosi et al. 1994). For example, the enzymes produced by some *Oenococcus oeni* are inhibited by glucose concentrations as low as 10g/L, while others show increased in hydrolytic activity (Grimaldi et al. 2001).

β-Glucosidase from *S. cerevisiae* performs optimally at pH of grape juice of 5 rather than the required pH, during vinification, of 3.0-3.5 (Günata et al. 1994). Acidic conditions in wine may denature and inhibit the enzyme hydrolases (Delcroix et al. 1994). Günata et al. (1994) found that the optimum temperature of yeast β-glucosidase is 45-50°C. The optimum temperature for the activities of plant β-glycosidases is generally 40-50°C (Lecas et al. 1991; Schreier and Schreier 1986) and they are mostly denatured at temperature above 50°C. Glycosidases from filamentous fungi are more heat resistant than those from yeast and plants.

A glucose concentration lower than 0.5% (w/v) inhibits β-glucosidase from *Hanseniaspora vinea* (Vasserot et al. 1989). *Debaryomyces hansenii* has an optimum enzyme production at a glucose concentration of 2-8%, while its activity was inhibited at concentrations above 9% (Rosi et al. 1997). Ethanol levels also affect the enzyme activity. The presence of 11-15% ethanol found in wine can affect the enzymatic activity. Aryan et al. (1987) have shown that grape and almond β-glucosidase lose up to 60% of their enzyme activity at an ethanol level of between 1-15%, but β-glucosidase produced by species of *Dekkera intermedia* (Blondin et al. 1983), *Candida molischiana* (Gonde et al. 1985) and *Hanseniaspora vinea*, as well as other fungi and yeast β-glucosidases, were not affected by the ethanol concentration in wine (Aryan et al. 1987, Delcroix et al. 1994).The most relevent source of β-glucosidase that can be used in the wine making is the one from fungi sources as it is tolerant to wine making conditions.

There are some inhibitors that affect β-glucosidase functioning. Glucono-δ-lactone is produced by grape-attacking fungi that can be found in wine must at concentrations of up to 2g/L and is one of the substances that highly inhibit β-glucosidase from plants (Heyworth and Walker 1962; Lecas et al. 1991). Other known inhibitors of β-glucosidase activity include castanospermine, deoxynojirimycin and methyldeoxynojirimycin (Ridruejo et al. 1989).

Metal ions such as Ag^{+}, Hg^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} inhibit plant and fungal glycosidase, with Ag^{+}, Hg^{2+} and Cu^{2+} being the strongest inhibitor ions of β-glucosidases (Ridruejo et al. 1989). Sodium dodecyl sulphate (SDS), chaotropic agent

(urea) and organic solvents such as dimethylformamide (DMF) also inhibit enzyme activity. Ethylene glycol and methanol were also found to inhibit β-glucosidase from maize and almonds (Esen and Gungor 1993).

2.9 APPLICATION OF THE β-GLUCOSIDASE ENZYME

β-Glucosidases have dual activity, namely the cleavage and synthesis of glycosidic bonds, and both play an important role in biotechnological applications, biological pathways, such as cellular signalling, the biosynthesis and degradation of structural and storage polysaccharides and the host-pathogen interaction. The application can be classified in two classes: (i) applications based on hydrolytic activity and (ii) applications based on synthetic activity.

2.9.1 Applications based on hydrolytic activity

The β-glucosidases contribute to cellulose hydrolysis because cellobiose inhibits both endo- and exoglucanases and it must be removed to allow the efficient and complete saccharification of cellulose (Bhatia et al. 2002). For example, in commercial cellulase preparations of *T. reesei*, the activity of β-glucosidase is low and it limits the rate and extent of glucose production. Supplementation with β-glucosidase is beneficial in single-stomachs such as pigs and chickens (Zhang et al. 1996), where cellulose degradation was increased through enzymes, leading to better utilisation of nutrients. The other candidates for hydrolytic attack by β-glucosidase are flavanoids and isoflavanoid glucosides. There are phenolic and, in phytoestrogen glucosides that occur naturally in fruits, vegetable, tea, red wine and soya beans, the aglycone moiety is released as a result of the hydrolytic activity of β-glucosidase (Matsuda et al. 1994). The β-glucosidase enzyme is important in the field of medicine as anti-tumour agents, in biomedical research and also in the food industry. β-Glucosidases are also associated with the removal of bitterness from citrus fruit juices by catalysing the hydrolysis of naringin (4,5,6-trihydroxyflavanone-7-rhamnoglucoside) to prunin (Roitner et al. 1984).

In food, the application of gellan exopolysaccharide produced by *Sphingomonas paucimobilis* is limited because of its high viscosity and low solubility. The intracellular β-glucosidase produced by *Bacillus* sp. was shown to catalyse the cleavage of the trisaccharide glycosyl-rhamnosyl-glucose, which is produced by the action of gellan lyase and extracellular glycosidases, to release glucose and rhamnosyl-glucose, thereby reducing the viscosity of gellan exopolysaccharide (Hashimoto et al. 1998). β-Glucosidase from bacterial sources such as *Cellovibrio mixtus* (Sakellaris et al. 1997), *Thermoanaerobacter brockii* (Breves et al. 1997) and *Thermotoga neopolitana* (Zverlow et al. 1997) acts as a laminaribiases property, which is significant in the production of laminarinase (endo-β1-3 glucanase) to act at the terminal step β-1-3 glucan hydrolysis

and release glucose from laminaridextrins and laminaribiose. This is important in the production of yeast extract and entails the conversion of algal biomass to ferment sugars.

β-Glucosidase is also associated with the enhancement of fruit and wine aroma through the liberation of monoterpene alcohols. The role of certain plant β-glucosidases is important in pigment metabolism and industrial purification. For example, dried saffron (*Crocus sativus*) flower florets are treated with β-glucosidase in order to isolate the precarthamine pigment. The deglycosylation of betacyanin (betalains) by β-glucosidase in *Beta vulgaris* is the first step toward the degradation of these compounds to release the bioactive cellular metabolites, which have anti-tumour activity and are used as natural food dyes in confectionary products (Zakharova and Petrova 2000). Figure 2.6 summarises the action of β-glucosidase on different types of glycosidic compounds, which results in the generation of useful products or properties (Bhatia et al. 2002).

2.9.2 Applications based on synthetic activity

The transferase activity of β-glucosidase can be used in the synthesis of a variety of compounds, such as oligosaccharides and glyco-conjugates. The role of these sugar-linked molecules is understood the best in biological and pharmaceutical science, where the applications are used on a large scale. Considerable progress has been made in the use of the enzymatic route of biosynthesis of these compounds. Synthesis with the use of chemical methods is often slow and non-specific and this hinders their application (Bhatia et al. 2002).

Reverse hydrolysis and transglycosylation have been used for biotransformation. Recently, the partially purified *Bgl* II enzyme of *Pichia etchellsii* expressed in *E. coli* was used for the biosynthesis of oligosaccharides by reverse hydrolysis and by the transglycosylation approach, and the yields were 14% and 8% respectively. Furthermore, the addition of dimethylsulfoxide (DMSO) further increases the yield by 10% in the transglycosylated approach. In the presence of DMSO there was an increase in the V_{max}/K_m of the synthetic reaction (Bhatia et al. 2002). Apart from primary, secondary and tertiary alcohols, monoterpenes and aryl alcohols, or even diols, may serve as acceptors of the glucosyl group in β-glucosidase-catalysed biotransformation. For instance, the glucosides of organosilicon alcohols synthesised by free and immobilised *P. furiosus* enzyme have potential applications as agrochemicals and drugs (Fischer et al. 1996).

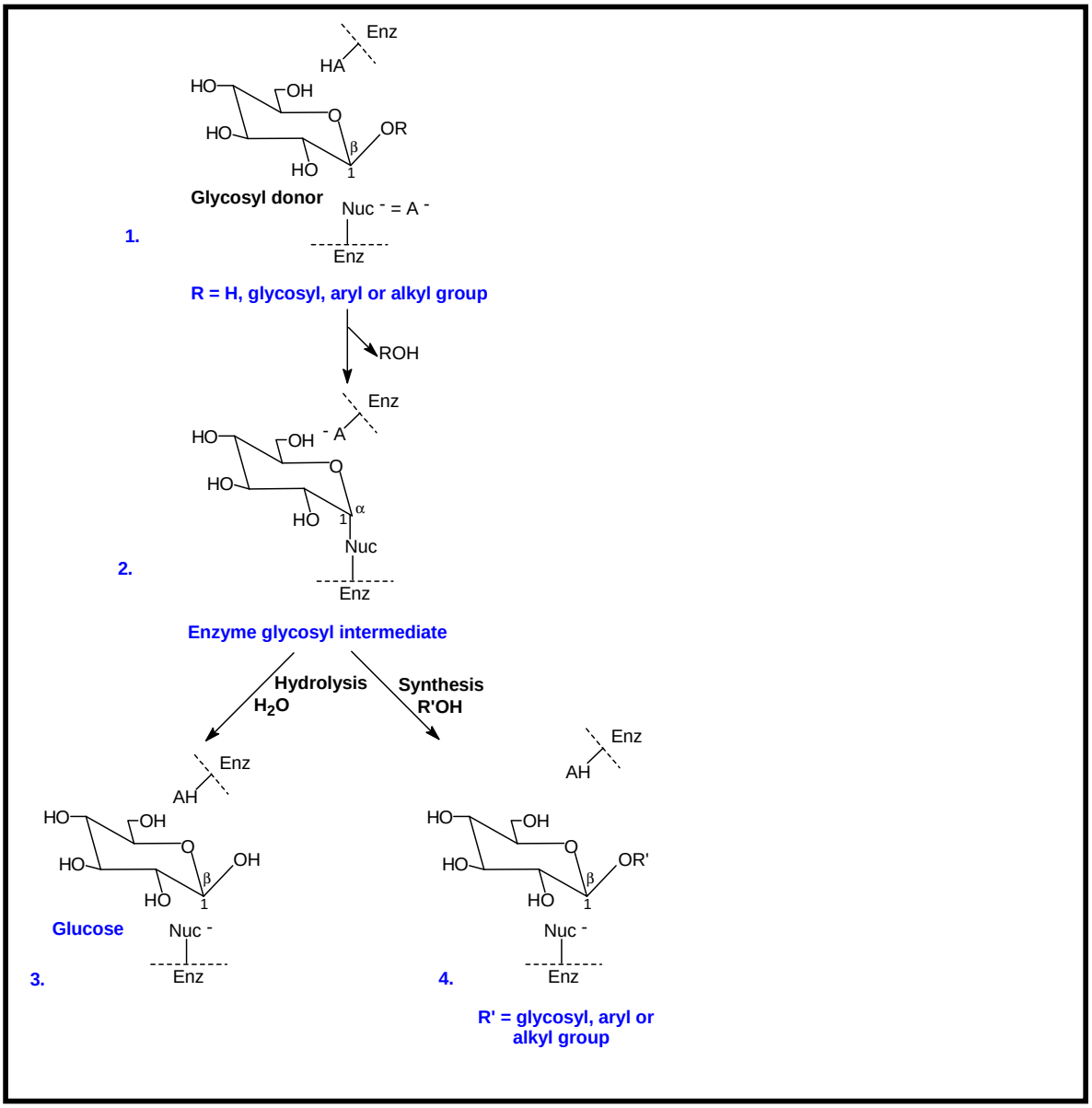

Figure 2.5 The proposed reaction mechanism of β-glucosidase. Nucleophilic attack by the enzyme functional group A- (1) leads to inversion of the anomeric configuration of the β-glucosidic bond in the enzyme-glycosyl intermediate (2). Subsequent reactions in the presence of H2O or sugars (aryl- or alkyl-glucosides) lead to the retention of the β-form, resulting in hydrolysis (3) or synthetic reactions (4) respectively (Bhatia et al. 2002).

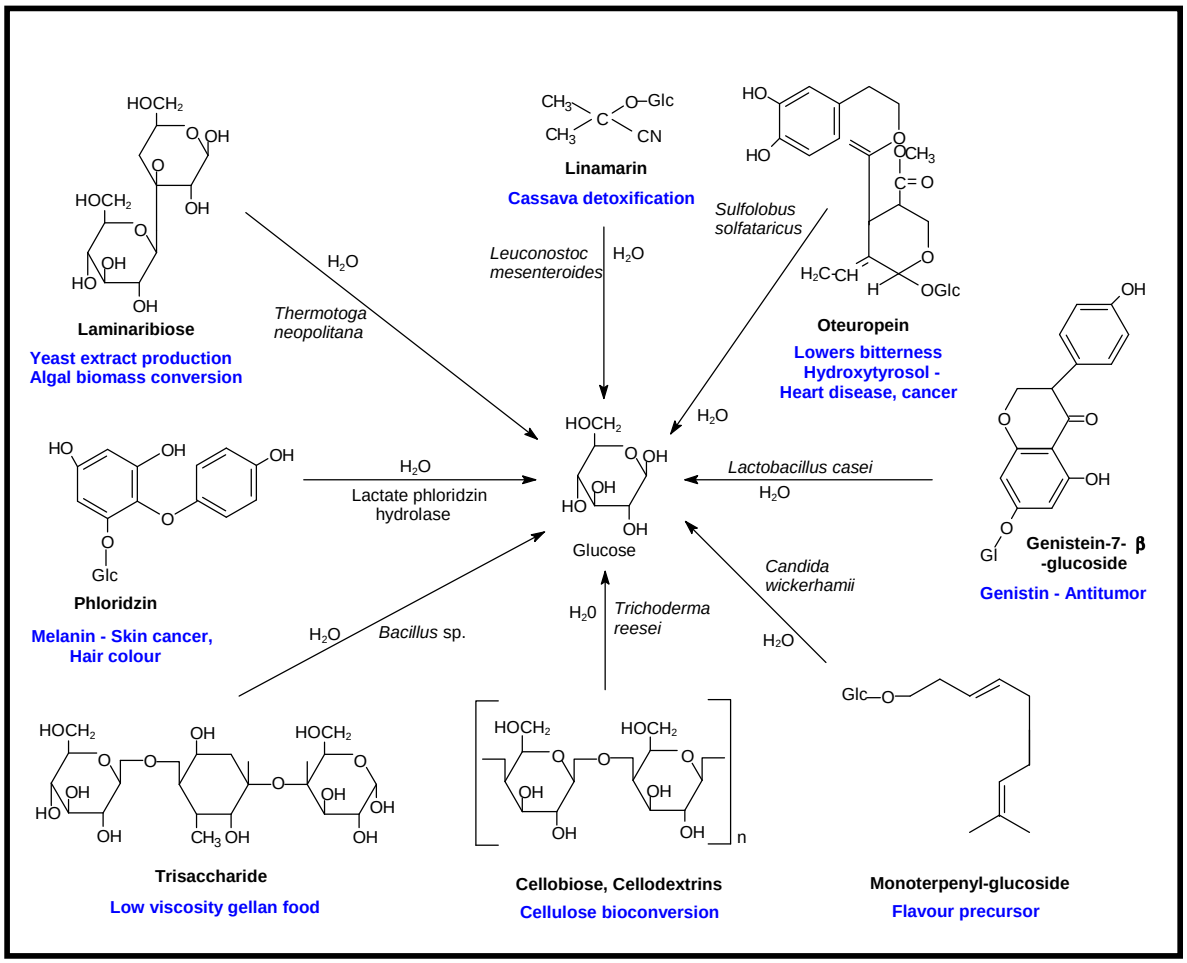

Figure 2.6 Applications based on the hydrolytic activities of β-glucosidase enzymes. The enzyme source for each reaction is shown on the arrow. The useful products or properties are indicated in blue (Bhatia et al. 2002).

2.10 IMPORTANCE OF CELL WALL ANCHOR PROTEIN

Ueda and Tanaka (2000) have shown the importance of using cell surface engineering. The *S. cerevisiae* strain reviewed in their study (Figure 2.7) is the first example of surface-engineered yeast in which active enzymes were targeted to the cell surface, resulting in cells with beneficial properties. This surface-engineered yeast strain was termed “arming yeast” because it displays enzymes that are also regarded as self-immobilised on the cell surface, with these features being passed on to daughter cells as long as the genes are retained by the cells. This system should further increase the status of *S. cerevisiae* as an attractive microorganism, because it can act as a whole-cell biocatalyst as a result of the surface expression of various enzymes. Heterologous protein can be expressed and immobilised on the surface of yeast. These proteins become covalently linked to the cell wall glucan, which makes them resistant to extraction. Yeast may have a long lifetime in industrial applications as a result of its tough cell wall (Schreuder et al. 1996).

The cell surface is a functional interface between the inside and outside of the cell. Surface proteins are responsible for most cell-surface functions, where they serve as cell-cell adhesion molecules, specific receptors, enzymes and transport proteins. Some of these surface proteins are found across the plasma membrane, whereas others are bound by non-covalent or covalent interactions to cell surface components (Ueda and Tanaka 2000).

The majority of cell-surface glycoproteins in yeast are non-covalently retained within the cell wall, either by interaction with the plasma membrane or by covalent linkage to the glucan structure (Klis 1994). Generally, the yeast cell wall proteins possess the following structure: an N-terminal hydrophobic sequence directing the protein into the yeast secretary pathway, an intracellular molecular Ser/Thr-rich spacer region and a C-terminal hydrophobic sequence responsible for cell wall anchoring (Klis et al. 1997). *In vivo*, the cell wall proteins enter the eukaryotic secretion pathway when they become post-translationally modified by N- and O-glycosylation and/or by attachment to the glycosyl-phosphatidyl-inostol anchor. After reaching the outer yeast cell surface, the proteins are incorporated into the cell wall via the C-terminal anchoring domain. Since yeast is a suitable expression system that tolerates certain cell surface modifications, it becomes more and more attractive as a host for the cell surface expression of foreign proteins in biotechnology, medicine and pharmaceutical applications (Breinig and Schmitt 2002). Cells have systems for anchoring surface-specific proteins and for confining surface proteins to particular domains on the cell surface (Ueda and Tanaka 2000).

One of the most suitable microorganisms used for food and pharmaceutical production is the yeast *S. cerevisiae*, which has ‘generally regarded as safe’ (GRAS) status. *S. cerevisiae* is a useful organism for the development of a cell-surface expression system. It can be used in genetic engineering because it can enable the folding and glycosylation of eukaryotic heterologous protein expression. It can easily be genetically manipulated and it can also be cultivated at a low cost to a medium and high density.

S. cerevisiae has a rigid, thick cell wall of about 200 nm that lies outside the plasma membrane. The two major components of the cell wall of *S. cerevisiae* are glucan and mannoprotein, which are present in roughly equal amounts. The glucan, which is made up of β-1,3- and β-1,6-linked glucose, is complexed with chitin to provide mechanical strength to the cell wall (Figure 2.8). The mannoproteins, which form the outer layer of the cell wall, are nearly glycosylated and determine most of the surface properties of the cell (Schreuder et al. 1996). Two types of mannoproteins are present in the cell wall of *S. cerevisiae*. Mannoprotein is loosely associated with the cell wall through a non-covalent bond and is extractable with sodium dodecylsulphate (SDS) and, if the isolated cell wall is solubilised by hot SDS, about 60 low-molecular-weight proteins are released. Glucans are released by β-1,3 or β-1,6 glucanase digestion of the glucan layer of the cell, but not by SDS extraction (Ueda and Tanaka 2000). There are many cell-surface proteins in yeast, for example Agα1, Aga1, Flo1, Sed 1, Cwp1, Cwp2, Tip1, Tir1 and

Srp2, all of which have a GPI anchor, which plays important roles in the surface expression of cell-surface proteins and are essential for the viability of yeast.

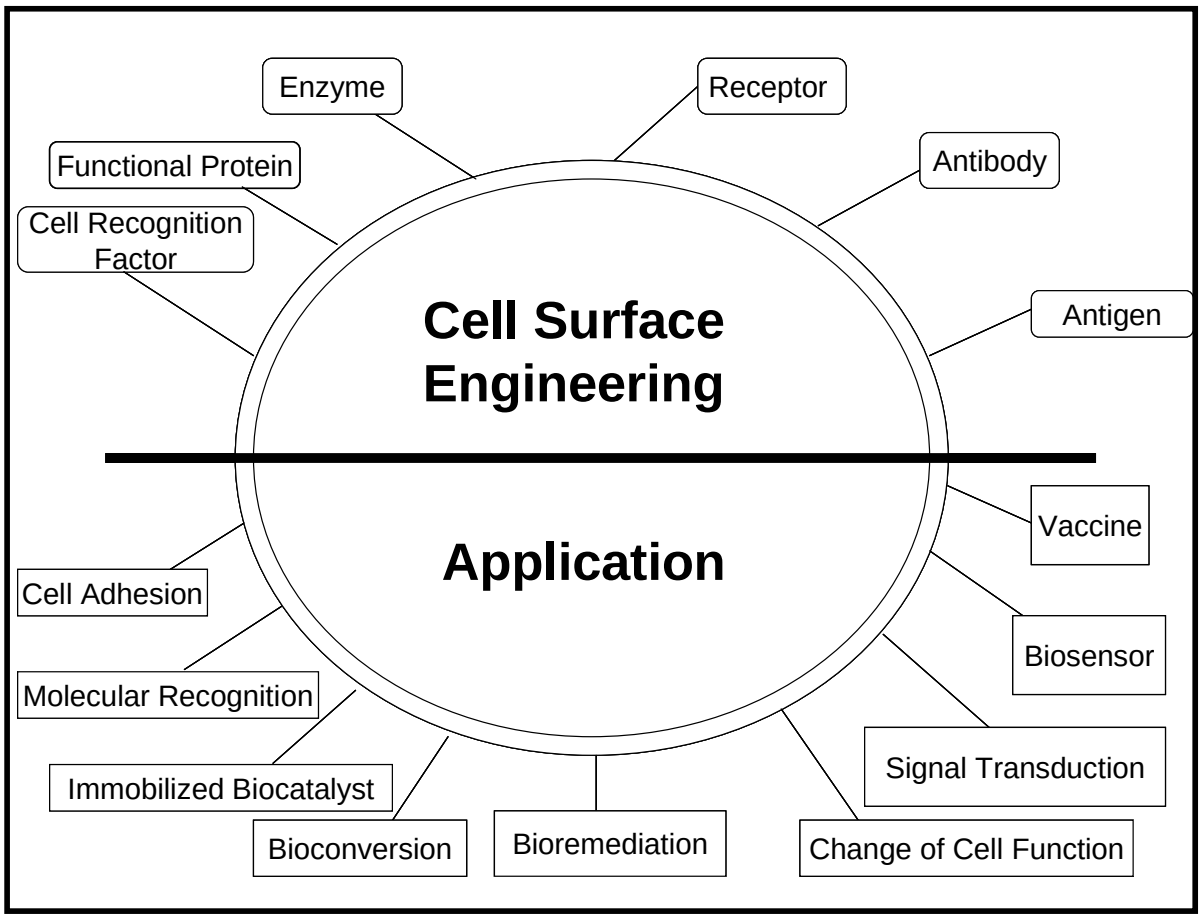

Figure 2.7 Arming yeast constructed by cell surface engineering and its application in biotechnology (Ueda and Tanaka 2000)

2.11 CONCLUSION

Winemaking is a microbial process that involves different yeast and lactic acid bacteria, and there are many enzymes involved in the fermentation process. Good wines are valued for having an intense colour and good flavour and aroma profiles. Monoterpenes are among the compounds that contribute to the aroma of wine. They can be found as either free aroma precursors, which are volatile and odorous, or as glycosidic precursors, which are non-volatile and odourless, with glycosidic precursors being more abundant than the free aroma ones.

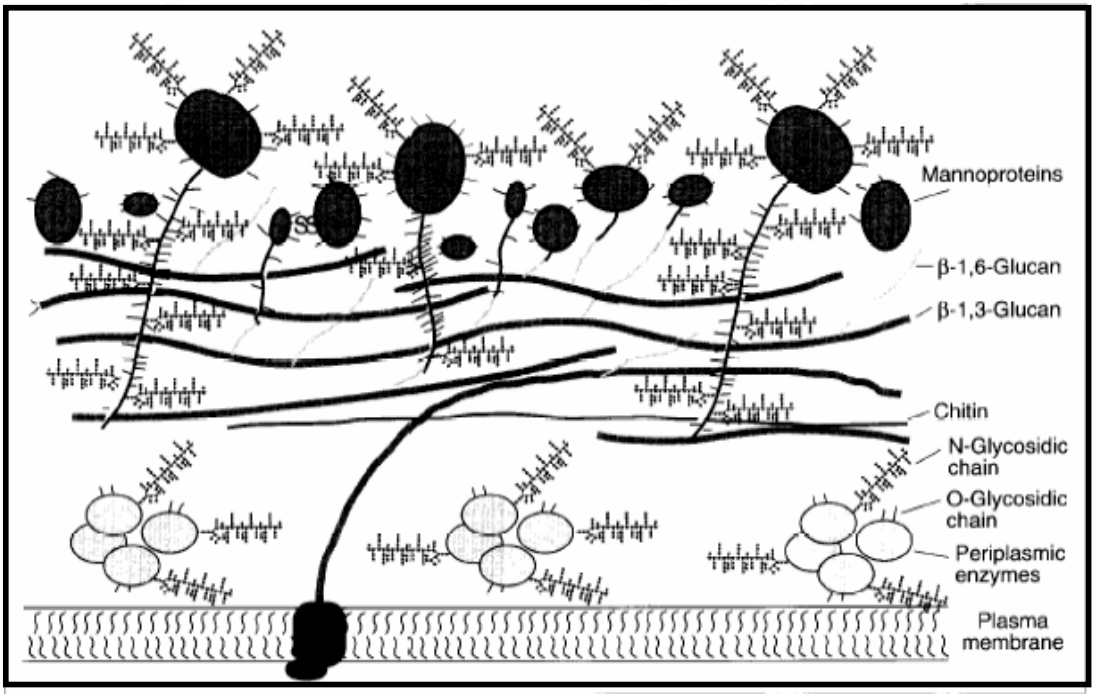

Figure 2.8 Composition and structure of the cell wall of S. cerevisiae (Schreuder et al. 1996)

It is now well established that glycoconjugated volatile compounds have an important potential for enhancing the flavour of grape juice and derived wine beverages. Flavour increase in juice and particularly in wine processing has become possible through the use of exogenous glycosidase. New challenges are opened through genetic engineering by constructing recombinant yeast strains. The use of recombinant yeast that will be able to unleash the glycoside pool will reduce the costs related to the use of commercial enzymatic cocktails with glycosidase activity among others. These enzymes are of importance because they have both hydrolytic and synthetic activity and a wide range of applications in the medical and biotechnological sectors. There are many sources of β-glucosidase, such as plants, yeasts, bacteria, fungi, etc. *S. cerevisiae* may have a long lifetime in industrial applications because of its high fermentation capacity and its rigid cell wall. Proteins can also be immobilised and expressed on the surface of *S. cerevisiae*, where the protein becomes covalently linked to the cell wall glucan, which makes it resistant to extraction. This characteristic can have important applications under winemaking conditions, because of the many proteolytic enzymes found in the grape must. A combined fermentation and β-glucosidase action can have far-reaching advantages for the wine industry.

This literature review has focused on the importance of β-glucosidase under winemaking conditions, on the types of monoterpenes, the structure of the glycosides, the method of hydrolysis of terpene glycosides, on the sources of this enzyme, its classification and mode of action, on factors affecting it, its application and the importance of the cell-wall anchor protein. However, more effort is still needed to optimise the expression and functioning of β-glucosidase in yeast.

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CHAPTER 3

RESEARCH RESULTS

Improving β-glucosidase activity in recombinant *Saccharomyces cerevisiae* strains

This manuscript is to be submitted for publication in
Food Microbiology

Improving β-glucosidase activity in recombinant *Saccharomyces cerevisiae* strains

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3.1 ABSTRACT

A portion of the aroma compounds (monoterpenes) presents in most white and red grape varieties are in a free volatile and odorous form, while others occur as glycosidically-bonded, non-volatile and non-odorous aroma precursors. Enzymes such as β–glucosidases can carry out the enhancement of wine aroma by hydrolysis of the glycosidic precursors from the grape. β-glucosidase occurs naturally in plants, yeasts, bacteria and fungi. Not all of these β-glucosidase enzymes can be used because of the harsh conditions of winemaking (low pH and temperature, and high ethanol and sugar concentrations). The β-glucosidase (*BGLA*) from fungal origin *Aspergillus kawachii* has been shown to perform well in winemaking conditions and was used in this study. *BGLA* gene was cloned, integrated and expressed with different expression cassettes in *Saccharomyces cerevisiae* W303-1A strain. Growth curve analysis was done on 2% cellobiose and the recombinant strains showed an increase in growth as compared to the reference strain W303-1A. The recombinant strain W16 showed the highest activity of 0.491 mM/mg of dry weight showing that it is able to cleave glycosidic bond efficiently of ρ-nitrophenyl-β-D-glucopyranoside (ρNPG) which was used as a substrate. However, W97, W98 and W24 did not show significant difference in β-glucosidase activity as compared to the wild type W303-1A. The kinetic characterization of the recombinant enzyme from W16 strain shows an optimum pH of 4 and temperature at 40˚C which is not ideal for winemaking conditions, however significant activity was obtained by the best performing stain (W16) in lower temperature and pH. There was a significant increase of monoterpenes released during Gewürztraminer must fermentation with W16 strain as compared to the reference strain, compounds such as citronellol had 2 fold increase, nerol had 4 fold increase, and geraniol had 20% increase.

Key words: *Saccharomyces cerevisiae*, β-glucosidase, Monoterpenes, Wine yeast.

3.2 INTRODUCTION

High quality wines are characterised by their colour, flavour and aroma profiles. Monoterpenes are one class of compounds that contribute to the aroma of the wine. Aroma is influenced by interaction between compounds derived from the grapes, those produced during fermentation and those produced during ageing. Chemical composition of wine is determined by factors such as grape variety, geographic and viticultural conditions, microbial ecology of grapes and wine-making practices (Cole and Noble 1997). Monoterpenes ratio can vary depending on the cultivar: in Muscat and Riesling the ratio between free aroma and glycosidically bound can vary between 1:5 respectively, and in Gewürztraminer variety it can vary up to 15 (Günata et al. 1988). Monoterpenes occur as either free aroma, volatile and odorous or as glycosidically bound, non-volatile and non-odorous (Williams et al. 1980). There are two methods that can be used to break down the glycosidic bond: the acidic or the enzymatic method. The acid method is disadvantageous because it modifies the terpenol extensively whereas the enzyme method causes only minimal changes. There are two steps involved in the enzyme method. Firstly, glucose is separated from the terminal sugar by a hydrolases (e.g. α-L-arabinofuranoside, α-L rhamnosidase or β–apiosidase) and secondly the bond between the aglycone and sugar is broken by β–glucosidase (Günata et al. 1988). When a disaccharide moiety consists of two glucose units only the action of β–glucosidase is needed to facilitate the complete reaction. The compounds bound to a sugar molecule are known as aglycone and in grape these may be aliphatic residues such as monoterpenes, sesquiterpenes, norisoprenoids or shikimic acid and metabolites such as phenols. The aglycone is always linked to β–D-glucopyranose (Abbott et al. 1993; Sefton et al. 1993, 1996; Winterhalter et al. 1990). In case of diglycosides the moiety is substituted with one of the following sugars: α-L-arabinofuranose, α-L-arabinopyranose, α-L-rhamnopyranose, β–D-glucopyranose, α-L-apiofuranose and α-L-xylopyranose (Sarry and Günata 2004).

β–glucosidase, also known as β-D-glucoside glucohydrolase (E.C 3.2.1.21), is a group of enzymes that hydrolyse a variety of glycosides, including alkyl- and aryl-β-D-glucosides (e.g. methyl-β-D-glucoside and ρ-nitrophenyl-β-D-glucoside) and also glycosides as cellobiose that contain only carbohydrates residues (Woodward and Wiseman 1982).

There are many sources of β–glucosidase present in nature such as plants, yeasts, bacteria and fungi. Few β–glucosidases can be used in wine making conditions such as low pH and temperature, high glucose concentration and high level of ethanol (Aryan et al. 1987; Woodward and Wiseman 1982). In this study a β–glucosidase from *Aspergillus kawachii* was used because it is glucose tolerant and stable at low pH values as compared to β–glucosidases from other origins (Aryan et al. 1987; Gunata et al. 1985). For instance, plant β–glucosidase is inhibited by glucose concentrations above 1% and is not active between pH 3 and 4 (Aryan et al. 1987), while the bacterial

enzymes have a disadvantage of being active only at high temperatures such as 65˚C (Ait et al. 1979).

The aims of this study were to clone, integrate and express *BGLA* from *Aspergillus kawachii* into *S. cerevisiae* W303-1A (reference strain). It was also proposed to test the enzyme activity with different expression cassettes, thus comparing if the presence of cell wall anchor domains played any role in the activity. Wine prepared from Gewürztraminer cultivar using the genetically modified yeasts were analysed for improvements in monoterpenol release.

3.3 MATERIALS AND METHODS

3.3.1 MICROBIAL STRAINS, PLASMIDS, MEDIA AND SCREENING PROCEDURES

All yeast and bacterial strains together with the plasmids used in this study are listed in Table 3.1. Transformants of *Escherichia coli* were grown at 37˚C in Luria Bertani (LB) broth and maintained on LB agar plates supplemented with 100 µg/ml ampicillin to maintain selection pressure (Sambrook et al*.* 1989). *S. cerevisiae* laboratory strain W303-1A and all the recombinant strains were grown at 30˚C either in rich YPD medium (1% yeast extract, 2% peptone and 2% dextrose), or YPC medium (1% yeast extract, 2% glucose and 2% cellobiose) or in a Synthetic Complete (SC) medium (0.67% Yeast Nitrogen Base, Difco Laboratories, 2% dextrose), supplemented with essential amino acids. For solid media, 2% agar was added. Yeast and bacterial cultures were maintained as 15% and 40% glycerol stocks respectively, at -80˚C.

3.3.2 CLONING OF β-GLUCOSIDASE GENE

The plasmid YAKA, containing the *BGLA* gene from *Aspergillus kawachii,* was used as template to amplify *BGLA* for all the constructs made. The MFα1BGLA-F and MFα1BGLA-R primers (Table 3.2) were used to amplify *BGLA* gene for cloning into the pCEL14 vector between the *MFα1* and the $PGK1_T$ sequences. The MFα1BGLA-F and BGLASTOP-R primers were used to obtain the *BGLA* gene for cloning into the pCEL23 vector between the *MFα1* and the CWP2 binding protein (Table 3.2). Polymerase Chain Reaction (PCR) was carried out in a PCR Express thermal cycler (Biometra®, Göttingen, Germany) for 15 cycles: 3 min denaturation at 95˚C; 1 min annealing, at 54˚C; 2 min extension at 72˚C. PCR products obtained from each reaction were initially cloned into a commercial vector, pGEM-T® easy (Promega, Mannhein, Germany) and their sequences were confirmed by sequencing. The 2529 bp fragment amplified from plasmid YAKA was digested with *Hin*dIII and ligated into linearised pCEL 14 vector and resulted in pCEL 16. The same fragment amplified from YAKA was digested with *Hin*dIII and *Bgl*II and ligated into linearised pCEL 23. This resulted in plasmid pCEL 24.

Table3.1 : Strains and Plasmids used in this study

	Relative genotype	Source/Reference
Strains		
Escherichia coli (DH5α)	*sup*E44 *ρlac*U169 (ϕ*80lac*ZρM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	GIBCO/Bethesda Research Laboratories
S. cerevisiae (W303-1A)	*MAT a leu 2-3,112; his 3-11,15; trp 1-1; ade 2-1; ura 3-1*	(Thomas and Rothstein 1989)
WYAKA	W303, *URA3 ::* YAKA	This study
W97	W303, *URA3 ::* pDLG 97	This study
W98	W303, *URA3 ::* pDLG 98	This study
W16	W303, *URA3 ::* pCEL 16	This study
W24	W303, *URA3 ::* pCEL 24	This study
Plasmids		
YAKA	Ap^R $PGK1_P$-XYL_S-*BGLA*-$PGK1_T$	IWBT,Stellenbosch.(SA)
pDLG 97	Ap^R $ENO1_P$-XYL_S-*BGLA-CWP2*-$ENO1_T$	(Van Rooyen et al. 2005)
pDLG 98	Ap^R $ENO1_P$-XYL_S-*BGLA-AGα1*-$ENO1_T$	IWBT,Stellenbosch.(SA)
pCEL 14	Ap^R $PGK1_P$-$MF\alpha1_S$-$PGK1_T$	(Gundllapalli et al. 2006)
pCEL 23	Ap^R $PGK1_P$-$MF\alpha1_S$-*CWP2*-$PGK1_T$	IWBT,Stellenbosch.(SA)
pCEL 16	Ap^R $PGK1_P$-$MF\alpha1_S$-*BGLA*-$PGK1_T$	This study
pCEL 24	Ap^R $PGK1_P$-$MF\alpha1_S$- *BGLA- CWP2*-$PGK1_T$	This study

Table 3.2 Primers used in this study

Primer	Oligonucleotide sequence[a]
MFα1BGLA-F	5'-**AAGCTT**GATGAATTGGCTTACTCC -3'
MFα1BGLA-R	5'-**AAGCTT**GTGAACAGTAGGCAGAG-3'
BGLASTOP-R	5'-**AGATCT**TAGTGAACAGTAGGCAGA-3'

[a] The restrictions sites are indicated in **bold**
AAGCTT- *Hin*dIII and AGATCT-*Bgl*II

3.3.3 TRANSFORMATION OF *S. CEREVISIAE*

The recombinant yeast integration plasmids pDLG 97, pDLG 98, pCEL 16 and pCEL 24 were linearised at the *Nco*I restriction site of the *URA3* marker and transformed into *S. cerevisiae* strain W303-1A using lithium acetate method (Ausubel et al. 1994). Transformants were selected on a Synthetic complete medium complemented with leucine, adenine, histidine and tryptophan. The transformants were confirmed by PCR and Southern blot. The evaluation of expression of different cassettes was carried out by Northern blot.

3.3.4 SOUTHERN BLOT HYBRIDISATION

Genomic DNA isolated from each of the recombinant strains and wild type was digested with restriction enzyme *Pvu*II and separated on agarose gel. The standard procedure for Southern hybridisation was followed according to the recommendations of the DIG Application Manual (Roche Biochemical Products, Mannheim, Germany). The blots were probed with 2.5kb DIG-labelled *BGLA* gene to confirm the integration of cassette at the *URA3* locus. DIG labelled DNA probe was amplified by using PCR primers MFα1BGLA-F and MFα1BGLA-R.

3.3.5 NORTHERN BLOT

Total RNA was extracted from the yeast cells by disrupting the cells with glass beads in the presence of phenol, chloroform and isoamylalcohol, as described by (Ausubel et al. 1994). The RNA was denatured by incubation with formaldehyde and then electrophoresed on 1.2% agarose containing formaldehyde. The RNA was transferred to a nylon membrane (Bio-Rad Laboratories, Hemel Hempstead, UK) by means of capillary blotting procedure, using Tris-acetate-EDTA blotting buffer. Blocking and hybridisation with DIG-labelled BGLA probe were performed essentially as described in the DIG application manual (Roche Biochemical Products, Mannheim, Germany). The autoradiography film was densitometrically analysed using Alpha Imager® gel document and image analysis system (Alpha Innotech, San Leandro, USA).

3.3.6 GROWTH CURVE ANALYSIS

Single colony of yeast strains W303-1A, W97, W98, W16 and W24 were inoculated into 10 ml YPD and incubated at 30˚C overnight. The recombinant yeast strains together with the reference strain were inoculated from a 18 h-old culture into a 250 ml Erlenmeyer flask containing 50 ml YPC to an initial OD_{600} of 0.1. The cultures were incubated at 30˚C in a shaker at 200 rpm and growth was monitored as optical density measurements (OD_{600}) using a spectrophotometer (UV-1601, UV-visible spectro-photometer, Shimazu) every 8 hrs until the stationary phase was reached. The experiment was done in triplicate and a Standard deviation of <5 were obtained.

3.3.7 β-GLUCOSIDASE ASSAY

A modified method of McMahon et al. (1999) was used to determine β-glucosidase activity. The activity of transformed strains and wild type were measured as follows. The yeast strains were grown in 50 ml YPD medium on a rotor at 30˚C for 40 h. β-glucosidase activity was determined by mixing 0.2 ml samples of the culture with 0.2 ml of 5 mM solution of ρ-Nitrophenyl-β-D-glucopyranoside (ρNPG; Sigma, St.Louis, MO, USA), ρNPG was dissolved in 50 mM Sodium acetate buffer pH 4. Incubation took place at 40˚C for 90 minutes. and the reaction was stopped by the addition of 1.2 ml of 1M sodium carbonate. The release of ρ-Nitrophenol (pNP) was determined by absorbance measure at 405 nm. Enzyme activity was expressed as mM/mg of dry

weight at optimum pH and temperature. The assays were performed in duplicates. Enzyme activities were determined for optimum temperature (10-70˚C) and optimum pH (2-10).

3.3.8 MICROVINIFICATION

Wine was made in triplicates from juice derived from Gewürztraminer cultivars. Gewürztraminer grapes was de-stemmed, crushed and then pressed. Overnight skin contact was given at 15˚C, followed by overnight clarification at 15˚C with half dose of pectinolytic enzyme, and 40 mg/L $S0_2$ was added. Standard analyses were done in unfermented must (titratable acid 2.65 g/L with 3 g/L tartaric acid, pH 3.53 and sugar of 25˚B). The reference yeast W303-1A and the 4 recombinant strains W97, W98, W16 and W24 were grown in 200 ml YPD for 2 days at 30˚C and then centrifuged to collect the biomass. The yeast was inoculated into 700 ml grape must to a final concentration of $3x10^6$ cells/ml and fermented in 750 ml bottles at 15˚C. The progress of fermentation was followed by measuring the decreasing bottles weight, and the alcoholic fermentation was considered complete when the weight of the bottles stabilized. When fermentation was complete, wines were racked off their lees and left at 4˚C to undergo tartarate stability.

3.3.9 GC-FID MEASUREMENT OF MONOTERPENES

Equipment model: Volumetric material with HP 6890 series gas chromatograph, fitted with splitless injector and automatic sampler 7683 was used with column: DB-FFAP, (60m ×0.32mm × i.d. 0.5µm film thickness). The chromatographic conditions were as follows:the carrier gas H_2 was used with a flow rate of 3 ml/min . The injector was used in splitless mode with a temperature of 220˚C and the injection volume was 1 µl. The detector temperature was 250˚C. The oven program was as follows: initial column temperature of 40˚C which was held for 12 minutes, and the temperature rise to 190˚C at 12˚C/min, then to 250˚C at 15˚C/min, and held for 2 min.

Method of volatiles extraction: 4 ml of dichloromethane was added to a C-18 cartridge (Hf Bond Elut LR-C18 OH, 500 mg, Varian) and eluted, then 4 ml of methanol was passed through the cartridge and finally 4 ml of artificial wine (wine stimulant) (distilled water, 12% ethanol, 2.5 g/L tartaric acid, pH 3.5 adjusted with NaOH) was eluted. It was ensured that the flow rate was not too high and the cartridge does not run dry. The wine sample of 50 ml complemented with 50 µl of a 2.5 g/L of 2,6-Dimethyl-6-hepten-2-ol solution in ethanol (internal standard), was passed through the column. The cartridge was washed with 4 ml of wine simulant again, and dried under vacuum for 15 min. The monoterpenes were eluted with 2 ml of dichloromethane into a tube. A spatula of Na_2S0_4 was added into the tube to absorb water. The recovered organic phase was transferred with a Pasteur pipette to a GC-vial and analyses were done under the chromatographic conditions as described above.

3.4 RESULTS AND DISCUSSION

3.4.1 Isolation, cloning and expression of *BGLA* gene

A gene encoding β-glucosidase (*BGLA* from *Aspergillus kawachii*) was amplified from plasmid YAKA using the sequence specific primers MFα1BGLA-F and MFα1BGLA-R (Table 3.2). The 2.5 kb PCR product was inserted between the $MF\alpha1_S$ and $PGK1_T$ of plasmid pCEL 14 (Table 3.1), thereby generating a yeast integrating plasmid containing *PGK1*$_P$-*MFα1*$_S$-*BGLA*-*PGK1*$_T$ cassette (pCEL 16). The same 2.5 kb PCR product was inserted between the *MFα1*$_S$ and *CWP2* region of pCEL 23 using the primers MFα1BGLA-F and BGLASTOP-R (Table 2), generating the integrating plasmid pCEL 24 containing *PGK1*$_P$-*MFα1*$_S$-*CWP2*-*BGLA*-*PGK1*$_T$ (Table 3.1). The resulting plasmids pCEL 16, pCEL 24, pDLG 97, and pDLG 98 were integrated separately into *URA3* locus of *S. cerevisiae* W303-1A. Transformants were selected on SC^{-ura} plates and positive were frozen at -80˚C in glycerol stocks 15% (v/v) for later use. The integrations were confirmed by Southern blot (Figure 3.1) and PCR (data not shown).The expected band sizes for different integration were observed. Northern blot was done to evaluate the expression of *BGLA* gene and all the intergrated plasmids were expressed (Figure 3.2).

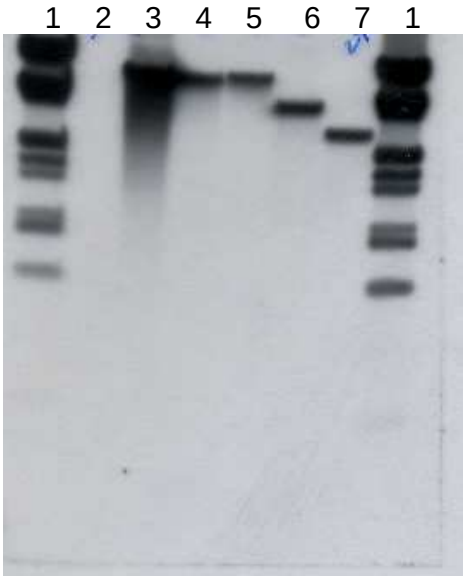

Figure 3.1: Southern blot was done by using Genomic DNA digested with *Pvu*II restriction enzyme. Line1, MW marker (*Eco*R1 and *Hin*dIII); line 2, Negative control (-) W303-1A; line 3, Positive control (+) WYAKA; line 4, strain W97; line 5, strain W98; line 6, strain W16; line 7, strain W 24.WYAKA was used as positive control because it was used as a template.

3.4.2 The physiological properties of the expressed β-glucosidase

Growth of the recombinant strains and wild type in rich media with cellobiose as a carbon sources was determined. An overnight YPD culture was used to inoculate to an OD_{600nm} of 0.1 into 50 ml of YPC. Growth at 30˚C was monitored and cell density was read in 8h intervals until the stationary phase was reached. Figure 3.3 shows the growth of yeast strains in 2% cellobiose. The recombinant strain W24 showed three times the cell density at the time-point 48 h when compared to the reference strain, whereas other strains showed even better growth on cellobiose. The strain W97 grew the most (OD_{600} 5) followed by the strains W16, W98 and W24. This shows the enhancement of β-glucosidase activity because cellobiose constitutes of a disaccharide subunit of cellulose, which is composed of two glucose molecules linked by a β (1→4) bond and all the strains that express the β-glucosidase will be able to hydrolyse the β (1→4) bond, thereby releasing the glucose monomers that can be used by the cells that reflected in the increase of cell density. Thus, the growth differences observed in cellobiose-containing media were as a result of β-glucosidase expression by the recombinant strains.

3.4.3 Enzyme activity, optimum temperature and pH

All the yeast strains were examined for their ability to produce β-glucosidase under standard assay conditions. The recombinant strain W16 produced the highest enzyme activity, more than fourfold (0.491 mM/mg) than the other strains. In fact, none of the other recombinant strains (W97, W98 and W24) showed any significant difference compared to the reference strain W303-1A (Figure 3.4). Recombinant strain W16 which showed higher activity was under *PGK1*$_P$-*MFα1*$_S$-*PGK1*$_T$, whereas the recombinant strain W24 which was expressed under the same conditions as W16 and linked to the cell wall anchor protein (CWP2) did not show activity when compared to the wild type. W97 and W98 expressed with *ENO1*$_P$-*XYL*$_S$-*ENO1*$_T$, and *CWP2* and *AGα1* anchor proteins respectively did not show an increase in activity. Hence linking the *BGLA* gene to the cell wall anchor protein in W303-1A background does not increase β-glucosidase activity. This can be due to the fact that the cell wall anchor domain causes conformational change and this causes the secreted enzyme to be unaccesible,thus why no much activity was observed in the other starins that are linked to cell wall anchor domain. The enzyme activity of the recombinant strains was further characterized for pH and temperature optimums. The optimum temperature was found to be 40˚C for the recombinant strains and the reference strain (Figure 3.5). There was a gradual increase in activity from 20 to 40˚C, which then decreased sharply in higher temperatures (Figure 3.5). Recombinant strain W16 showed about 20% relative activity at 10˚C, while the other recombinant yeast strains W97, W98 and W24 showed about 10% activity. The reference strain W303-1A showed no activity between 10˚C and 30˚C. In the study done by (Riou et al*.* 1998), it was reported that β-glucosidase was stable at temperatures up to 45˚C and almost inactive at temperature above 60˚C. The optimum pH determination was done in a range between 2 and 9, at 40˚C for 90 min. All the strains were found to

have an optimum activity at pH 4 (Figure 3.6). The best performing strain W16 was active also at low pH whereas the other recombinant strains show activity from pH 3. The reference strain was only active at pH 4 and 5 (Figure 3.6). Sternberg (1976) reported that β-glucosidase from *Aspergillus* sp. had an optimum pH between 4 and 5. Belancis et al. (2003) found that β-glucosidase from *S. cerevisiae* showed an optimum temperature of 40˚C. Recently, Villena et al. (2007) also reported that the optimum temperature for β-glucosidase was at 40˚C and optimum pH 4. At higher conditions, the activity decreased sharply.

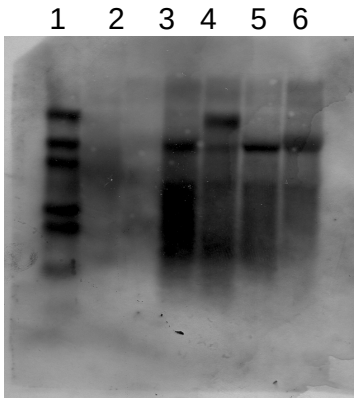

Figure 3.2: Northern blot was done by using RNA isolated from genomic DNA. Line1, MW marker (RNA marker with 1000 bp, Low range); line 2, Negative control (-) W303-1A; line 3, strain W97; line 4, strain W98; line 5, strain W16; line 6, strain W 24.

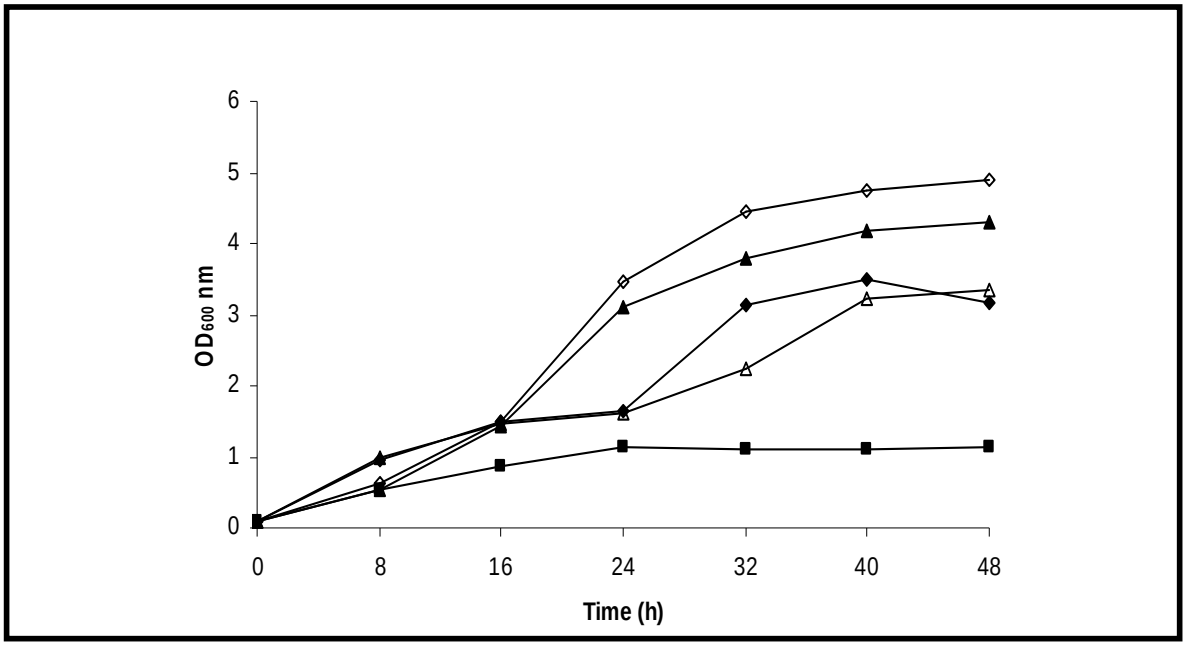

Figure 3.3: Growth curves of the reference strain W303-1A (■) and the recombinant strains W97(◇), W98(◆), W16(▲) and W24(△) in media containing 2% Cellobiose (YPC). The values represented are averages of two individual experiments performed in triplicates (Standard deviation <5%).

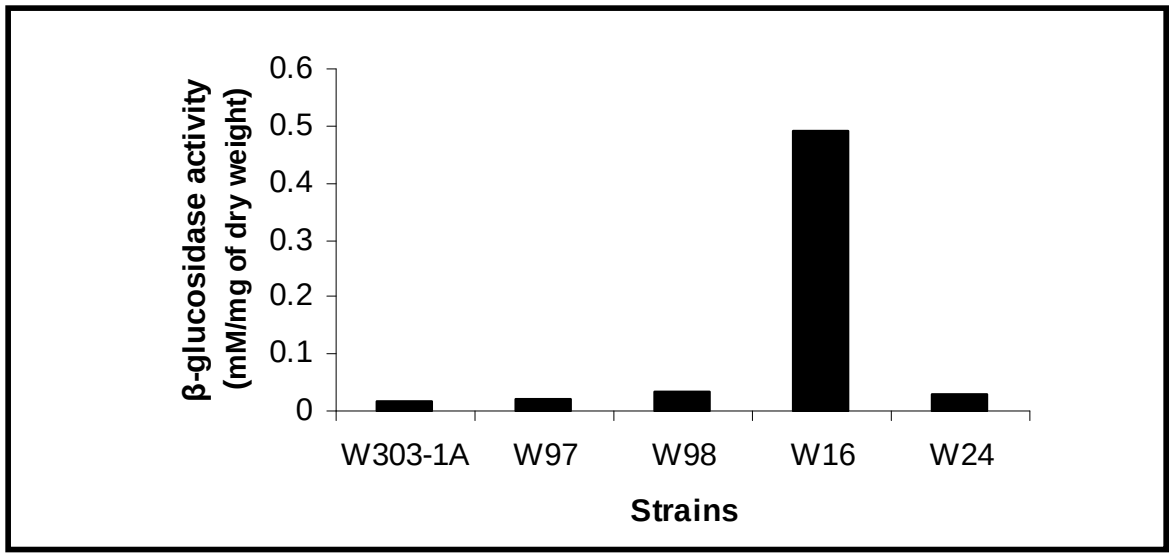

Figure 3.4: β-glucosidase activity of the recombinant strains (W97, W98, W16 and W24) and the reference strain W303-1A. The values represented are averages of two individual experiments done in triplicates (Standard deviation<10%).

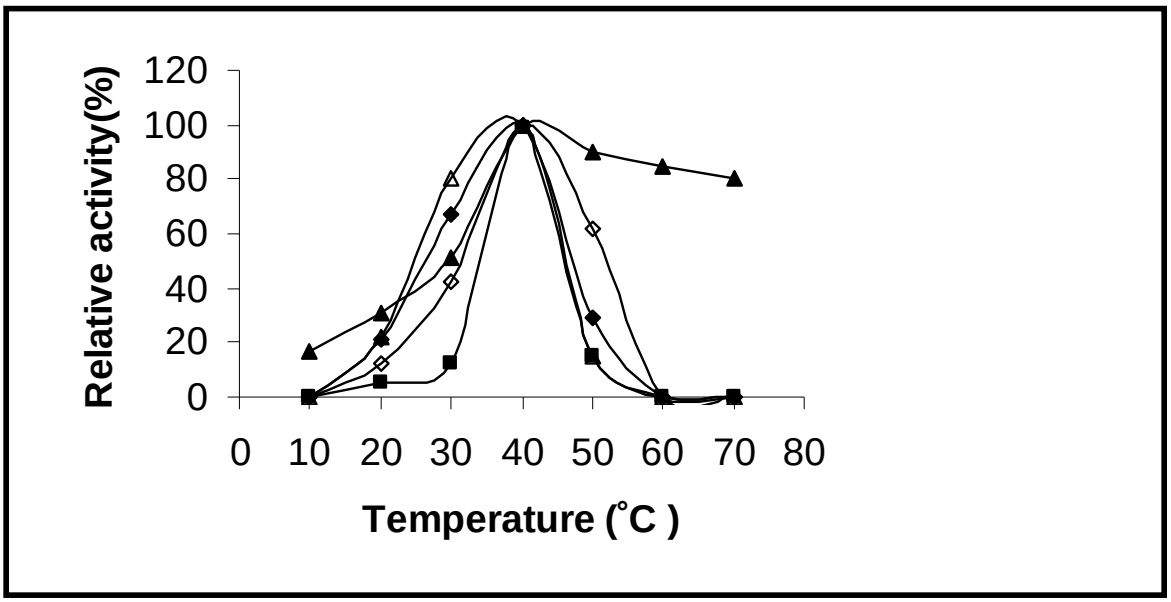

Figure 3.5: Temperature optimum of the β-glucosidase from the reference strain W303-1A (■), and the recombinant W97 (◇), W98 (◆), W16 (▲), W24 (△) strains. The scale of relative activity (%) indicates the percentage of experimental value at various temperatures relative to the maximum. The values represented are averages of experiment done in triplicates (Standard deviation<5%).

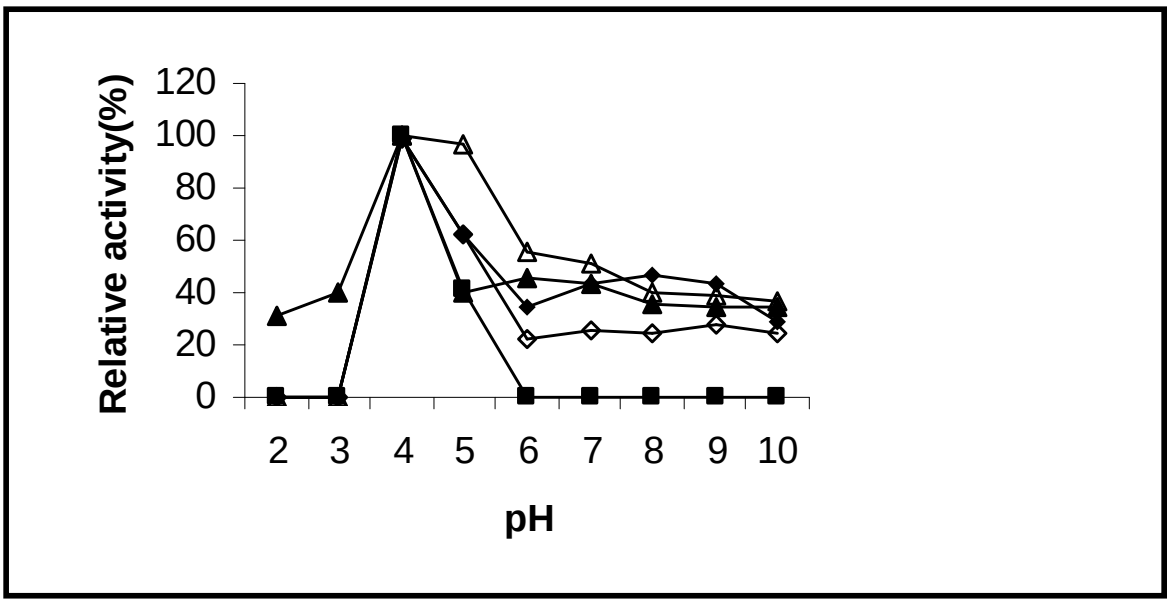

Figure 3.6: pH Optimum of the β-glucosidase from the parental W303-1A (■) and the recombinant W97 (◇), W98 (◆), W16 (▲) and W24 (△) strains. The scale of relative activity (%) indicates the percentage of experimental value at various and pH relative to the maximum. The values represented are averages of experiment done in triplicates (Standard deviation<5%).

3.4.4 Monoterpenes analysis

In this study, Gewürztraminer cultivar was used because it has more potential of the glycosidical precursors compared to other cultivars. Overnight skin contact was done to assist in the extraction of glycoside precursors (Cabaroglu et al. 2003). GC-FID analyses were done for both recombinant strains and reference strain W303-1A. There was an increase in the amount of all the monoterpenes produced by recombinant strain W16 as compared to all the other recombinant strains and reference strain W303-1A (Figure 3.7). Recombinant strain W16 produced more than 4 fold the amounts of nerol (85.6 µg/L), 2 fold citronellol (50.7 µg/L) and 20% increase geraniol (127.8 µg/L) than the reference strain. This strain also showed an increase in the levels of other monoterpenes such as linalool and α-terpinol but it was not significant as compared to the three mentioned above. W16 expressed *BGLA* under $PGK1_{P\&T}$, with $MF\alpha1_S$, has shows a significant increase in the amount of monoterpenes released. There was no significant increase in the amount of monoterpenes produced by the recombinant strains W97, W98 and W24 as compared to the reference strain. When *BGLA* is linked to the cell wall anchor protein it does not show any difference as compared to the wild type. This might be because β-glucosidase is selective to specific aglycones (Williams et al. 1995). Günata et al. (1985) showed that β-glucosidases from grapes acts on precursors that have a primary alcohol as aglycone, such as citronellol, geraniol but do not cleave glycosides bonding tertiary alcohol such as linalool and α-terpinol. A similar observation was made in this study even though the source of β-glucosidase differ as there was an increase in the amount of nerol, geraniol by W16 as compared to linalool and α-terpinol monoterpenes.

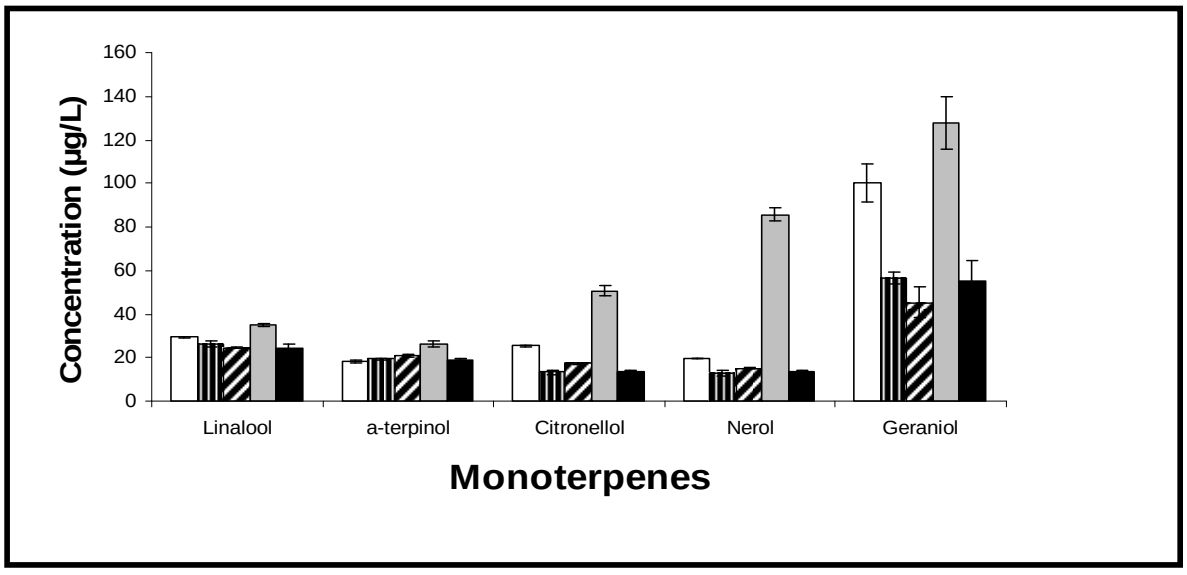

Figure 3.7: Analysis of Monoterpenes produce by the parental strain W303-1A (White bars) and the different recombinant strains (W97 - bars with vertical lines, W98 - bars with slanted lines, W16 - gray bars and W24 - black bars). (The values represented are averages of three individual experiments done in triplicates (Standard deviation<10%).

3.5 CONCLUSION

Based on the results from this study, *BGLA* has been successfully expressed in *S. cerevisiae*. The importance of promoters and terminator together with the secretion signal with which the gene was expressed has also been determined. The engineered W16 strain showed increase in β-glucosidase activity in all of the evaluated conditions. This recombinant strain that expressed the *BGLA* gene under $PGK1_P$ and $PGK1_T$ regulation and *MFα1* secretion signal expression show higher activity as compared to *BGLA* under the same promoter, secretion signal, terminator and also CWP2. The involvement of different cell wall anchor proteins (*CWP2,* and *AGα1* respectively) when *BGLA* was expressed by $ENO1_{P\&T}$ with the XYL_S secretion signal did not show any significant increase in enzyme activity in W303-1A background. There was also an enhancement on the amount of monoterpenes - citronellol, nerol and geraniol - produced by recombinant strain W16 as compared to reference strain W303-1A. Thus, the expression cassette integrated in strain W16, which has shown the best activity and monoterpene enhancement could be expressed in an industrial wine yeast strain and evaluated for improved aroma profile. Similarly, the cassette containing β-glucosidase could be expressed together with an enzyme such as α-arabinofuranosidase, which is involved in the first step of enzymatic hydrolysis of the glycosidic bonds. Screening for and expressing β-glucosidase that is more active at low temperatures could produce better results. Such studies expressing both α-arabinofuranosidase and β-glucosidase together are currently being done in the Institute, which will probably increase the amount of monoterpenes released.

3.6 ACKNOWLEDGEMENTS

The authors would like to thank the National Research Foundation (NRF), South Africa, South African Wine Industry (Winetech) and University of Stellenbosch for financial assistance.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

4.1 CONCLUDING REMARKS AND PERSPECTIVES

Wine is one of the complex beverages known to man. However a good wine is determined by its colour, flavour and aroma. Varietals aroma is determined by volatile compounds such as monoterpenes, norisoprenoids and benzene derivatives, which are naturally present in the wine (Fia et al. 2005; Williams et al. 1982; Günata et al. 1985). The aroma of the wine is influenced by many factors such as grape-derived compounds that exist as free volatiles and sugar bound glycosides (Abbott et al. 1993; Wiliams et al. 1982). The chemical composition of the wine is determined by factors such as grape variety, geographic position, viticulture conditions, microbial ecology of the grape and the wine-making practices (Cole and Noble 1997). Also microorganism can play an important role to determine the chemical composition of wine because they affect the grape prior to harvest and during fermentation (Nykänen 1986; Lambrechts and Pretorious 2000).

Monoterpenes is one of the compounds that contribute to the aroma of wine. Among the monoterpenes linalool, geraniol, nerol, α-terpinol and citronellol are more abundant due to their low sensory threshold (Williams et al. 1982; Günata et al. 1985). The monoterpenes occurs as either volatile, odorous and free volatiles or as non-volatile, non-odorous and glycosidically bound. In grapes of cultivars such as Gewürztraminer they ratio between free and glycosidically bond aroma compounds is about 15 to 1, whereas in other cultivars such as Muscat and Riesling the ratio is about 1 to 5 (Günata et al. 1988). Two methods can be used to unleash the pool of glycosides bonds and enhance the flavour and aroma of the wine, is either acidic method or the enzyme method. Enzyme method is mostly preferred, as it does not modify the monoterpenol (Rapp and Mandery 1986). There are many sources of β-glucosidase (plants, yeasts, bacteria, fungi, etc) but most β-glucosidase cannot be used in wine-making conditions of low pH and temperature, high ethanol and glucose as this enzyme become inactive (Aryan et al. 1987; Woodward and Wiseman, 1982). β-glucosidase from fungal origin especially *Aspergillus* sp are tolerant to wine making conditions (Sternberg 1976). The use of commercial enzymes in wine making is seen by many wine makers as unnatural and in can be costly in large cellars. This has lead to the construction of *S.cerevisiae* strain that will be able to produce β-glucosidase during wine making thereby liberating aroma compounds without the use of commercial exogenous enzyme preparation. This can be done by cloning A*spergillus kawachii* β-glucosidase gene into *S.cerevisiae*, as it was done in this study.

Chapter 1 of this thesis had emphasis on monoterpenes and the role they play in the aroma, flavour of the wine. As mentioned above, a small fraction of monoterpene occur as volatile, odorous and free whereas a big fraction occur as non-volatile, non odorous and glycosidically bond (Williams et al. 1982; Günata et al. 1985). This chapter shows how this glycosidic pool can be released to enhance the aroma. This can be done by the use of enzyme method; it also shows a broad overview of the enzymatic properties of some β-glucosidase from sources such as plant, yeast and bacteria.

The first section of Chapter 2 introduce the importance of enzyme in wine making, as well the functioning of β-glucosidase and also its importance in wine making and how it enhance the aroma of the wine. It also focuses on the type of monoterpenes and the structure in which the glycosidically bond compounds occur and on the hydrolysis of these bonds.

The second part of Chapter 2 focuses on different sources of β-glucosidase. Not all β-glucosidase from different origins are ideal for wine making conditions, an ideal β-glucosidase must be functional in low pH and temperature as well as in high sugar and ethanol (Aryan et al. 1987; Günata et al. 1990). In this section various sources of β-glucosidase were evaluated to determine which enzyme sources can successfully be used in the above harsh wine making conditions. The last part of Chapter 2 focuses on the classification and mode of action of this enzyme (Bhatia et al. 2002). There are many factors that makes this enzyme disfunctional, factors such as some metal ions, dimethylformamide, Glucono-δ-lactone, etc (Ridruejo et al. 1989). β-glucosidase have dual activity, the synthesis and cleavage of glucosidic bonds, these two mechanism plays an important role in biotechnology, also the importance of this enzyme in medical, industry and food sector was included in this chapter (Bhatia et al. 2002). The last concept discussed in this chapter was the importance of the cell wall anchor protein in biotechnology (Ueda and Tanaka, 2000).

Chapter 3 focuses on the cloning of the *BGLA* gene into pCEL 14 and 23 respectively and resulted into pCEL 16 and 24, together with pDLG 97 and 98 were integrated in URA3 marker of W303-1A and resulted in W97, W98, W16 and W24. All the recombinant strains and wild type W303-1A were grown in 2 % YPC and all the recombinant strains grow higher than the wild type and this shows that the recombinant strains have better functional β-glucosidase than the wild type. The enzyme activity was also measured using ρ-nitrophenyl-β-D-glucopyranoside as a substrate and it was found that W16 has about 4 fold more enzyme activity as compared to W97, W98 and W24, whereas W97, W98 and W24 did not show any significant increase as compared to the wild type. Enzyme characterization was done for optimum pH and temperature; it was found that BGLA from *Aspergillus kawachii* has an optimum pH at 4 and temperature at 40˚C, with W16 showing activity at lower pH as compared to the rest.

At the end all the recombinant strains together with W303-1A were tested in wine making conditions, were small scale fermentation was done using Gewürztraminer cultivar, and W16 shows the increase in the amount of citronellol, nerol and geraniol. The amount of linalool and α-terpinol did not show signification increase as compared to the wild type, also W97, W98 and W24 did not show any significant difference as compared to the wild type. From the data obtained from this study it clearly shows that the cell wall anchor domain does not increase the activity of β-glucosidase in the W303-1A background, whereas it shows that the expression on BGLA under $PGK1_P$ and $PGK1_T$ regulation and *MFα1* secretion signal expression is better as compared to the recombinant strains that had cell wall anchor proteins, hence the was no significant increase in the amount of monoterpenes released.

Future work in this study will be to express W16 in an industrial strain. Also to express the β-glucosidase together with one of the enzymes like arabinofuranosidase that is involved in the first step of the glycosidic bond hydrolysis.

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